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13. ABSTRACT *(Maximum 200 Words)*

wnt genes encode a large family of secreted signaling molecules essential for development and oncogenesis. wnt-1, the founding member of the wnt gene family, was initially identified as an oncogene. Ectopic wnt-1 expression causes mammary tumorigenesis in mice, providing a potential model for human breast cancer. However, the cell surface receptor (or receptors) that mediates Wnt-1 signaling had not been identified, and the molecular and biochemical nature of the Wnt signaling pathway was not fully understood. In a research supported in part by this Career Development Award, we identified a member of the LDL receptor-related protein (LRP) family, LRP6, as a Wnt-1 co-receptor, and a potent Wnt signaling antagonist, Dickkopf-1 (Dkk-1), as a ligand for LRP6. We also isolated Daam1, a Dishevelled-interacting protein, as a key molecule connecting Wnt signaling to cell morphological behaviors. These results have important implications in our understanding of Wnt-1 signaling in development and oncogenesis.

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FOREWORD

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Xi He 7/30/02

PI - Signature Date

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Introduction

Cell-cell communication plays a central role in animal development and tumor formation. Wnt signal transduction is a key part of this cell-cell communication network and is essential for establishing the basic vertebrate body plan and for maintaining human tissue homeostasis. Abnormal Wnt signal transduction causes mouse mammary tumors and several human cancers, including colorectal cancer, melanoma and possibly breast cancer (1). The mechanism of Wnt signal transduction during embryogenesis and tumor formation is not well understood.

Using a combination of molecular, biochemical and embryological techniques and supported in part by a Career Development Award from the DOD Breast Cancer Research Program, my colleagues and I have been trying to elucidate the molecular mechanism of Wnt signal transduction in Xenopus (frog) embryos during their development and in human cells in culture. I proposed two specific aims to address two critical questions in Wnt signaling: 1) What is the receptor mediating Wnt-1 function? 2) How does the Dishevelled protein, which is an essential Wnt signaling component, transduce Wnt signal? In the last 4 years, we have made significant progresses towards these two aims. Our results help to elucidate the molecular mechanism of Wnt signal transduction, and should shed light on the oncogenic functions of this important cell growth regulatory pathway.

Body

Research progresses in this section are organized according to the two tasks described in the original proposal, and are also discussed in the context of initial Statement of Work (SOW).

Task1. To identify the receptor(s) for Wnt-1 in the mammary gland that mediates Wnt-1 oncogenic functions (months 1-30).

1). Identification of a LDL receptor-related protein, LRP6, as a Wnt co-receptor

Tamai, K., Semenov, M., Kato, Y., Spokony, R., Liu, C., Katsuyama, Y., Hess, F., Saint-Jeannet, J. P., and He, X. (2000). LDL-receptor-related proteins in Wnt signal transduction. **Nature** 407, 530-535.

The Wnt family of secreted signaling molecules play essential roles in embryogenesis and tumorigenesis. The Frizzled (Fz) family of serpentine receptors have been shown to function as Wnt receptors, but how Fz proteins transduce Wnt signaling is not understood (1). Our work suggest that a member of the mammalian low-density lipoprotein receptor (LDLR)-related protein (LRP) family functions as a co-receptor for the Fz protein in Wnt signal transduction.

The Drosophila *arrow* locus defines a novel segment polarity gene whose mutant phenotype resembles that of the *wingless* (Drosophila Wnt-1) mutation (3). *arrow* encodes a transmembrane receptor homologous to two members of the mammalian LRP5 and LRP6 (2). We have investigated whether/how LRP6 is involved in Wnt signaling in Xenopus embryos. We found that ectopic expression of LRP6 activated Wnt signaling, inducing Wnt responsive genes, dorsal axis duplication and neural crest formation. LRP6 also exhibited strong synergy with Wnt or Fz in the induction of Wnt responsive genes. We also showed that a LRP6 mutant lacking the carboxyl cytoplasmic domain was unable to mediate Wnt signaling, but blocked Wnt or Wnt-Fz induced gene activation and neural crest formation. Co-injection experiments with various cytoplasmic components demonstrated that LRP6 functioned upstream of Dishevelled protein in Wnt responding cells. Finally, we showed that the extracellular domain of LRP6 bound Wnt-1, and associated with Fz in a Wnt-1-dependent manner. Our results suggest that LRP6 is a component of the Wnt receptor complex.

2). Identification of Dickkopf-1 as a ligand for Wnt co-receptor LRP6

Semenov, M. V., Tamai, K., Brott, B. K., Kuhl, M., Sokol, S., and He, X (2001). Head inducer Dickkopf-1 is a ligand for Wnt co-receptor LRP6. **Current Biology** 11, 951-961.

Although we demonstrated that LRP6 forms a Wnt induced complex with Fz, the significance of Fz-LRP6 complex in Wnt signal transduction was not fully established.

Our recent studies of a novel Wnt signaling antagonist provide strong evidence for the critical role of the Fz-LRP6 complex in Wnt signal transduction.

Dickkopf-1 (Dkk-1) is a signaling molecule secreted from the vertebrate head organizer and induces head formation by blocking Wnt signaling (3, 4). How Dkk-1 antagonizes Wnt function was unknown. We identified that Dkk-1 is a high affinity ligand for LRP6, and inhibits Wnt signaling by preventing Fz-LRP6 complex formation. Distinct from characterized Wnt-binding antagonists, Dkk-1 neither binds Wnt or Fz, nor does it affect Wnt-Fz interaction, but Dkk-1 binding to LRP6 prevents Wnt-LRP6 interaction. Dkk-1 function in head induction and Wnt signaling inhibition strictly correlates with its ability to bind LRP6 and to disrupt the Fz-LRP6 association. We also found that LRP6 is a Wnt co-receptor that appears to specify Wnt/Fz signaling to the β-catenin pathway, and Dkk-1 may be a specific inhibitor for Wnt/β-catenin signaling. Our findings suggest that Wnt-Fz-LRP6 complex formation, but not Wnt-Fz interaction, triggers Wnt/β-catenin signaling. These results provide a molecular basis for Dkk-1 function in head induction, reveal a novel mechanism for Wnt signal modulation, and highlight the critical role of Fz-LRP6 complex in Wnt/β-catenin signal transduction.

Thus with regard to Task 1, we successfully found that LRP6 is an essential part of the Wnt-1 receptor complex, most likely functions as a Wnt-1 co-receptor. Further, our study revealed a novel and intricate regulation of LRP6 co-receptor by Wnt antagonist Dkk-1. I should note that the initial emphasis of Task 1 was to identify which Frizzled (Fz) receptors function as Wnt-1 receptors (Tasks 1a-1f). This was because the existence of

LRP6 as a Wnt-1 receptor component was not known and very unexpected at the time of the proposal. Although we initially worked around Task 1a and 1b as reported in previous annual reports, our success with these tasks was very limited. Like many other scientific discoveries, interesting new directions would sometimes turn up in the most unexpected places. LRP6 was such a case, and thus we have performed research on LRP6 and Dkk1, neither of which was mentioned (or known to be relevant) in the original proposal. In short, we did not follow through Tasks 1c-f, but we added and successfully finished some other important tasks that are essential for the overall achievements of our Task 1.

Task 2. To identify molecules that associate with the Dsh protein and function between the Wnt-1 receptor and GSK-3 in Wnt signaling (months 1-48).

1). Understanding Dishevelled function in Wnt regulation of cell polarity and identification of Daam1 (*D*ishevelled-*a*ssociated *a*ctivator of *m*orphogenesis)

Habas, R., Kato, Y., and He, X. (2001) Wnt/Frizzled activation of Rho regulates vertebrate gastrulation and requires a Novel Formin Homology Protein Daam1. **Cell** 107, 843-854.

Wnt signaling via the Frizzled (Fz) receptor activates multiple transduction pathways in embryo development and tissue homeostasis. While Wnt/Fz activation of the b-catenin pathway controls cell fate and proliferation, Wnt/Fz signaling also governs planar cell polarity and cell movements via a distinct pathway. The molecular nature of Wnt signal transduction that regulates cell polarity remains poorly defined.

We found that in mammalian cells and during Xenopus gastrulation, Wnt/Fz signaling, via the cytoplasmic protein Dishevelled (Dvl), directly activates Rho, and this Rho activation is a specific result of Wnt cell polarity signal transduction independent of b-catenin signaling and strictly correlates with gastrulation movements. We used the yeast two-hybrid screen to identify Daam1 (Dishevelled-associated activator of morphogenesis), a novel Formin homology protein that binds to both Dvl and Rho and mediates Dvl-Daam1-Rho complex formation in response to Wnt signaling. We further showed that a constitutively active form of Daam1 activates Rho, whereas dominant negative mutant forms of Daam1 disrupt Wnt-induced Dvl-Daam1-Rho complex formation, and block Rho activation by Wnt/Fz/Dvl but not by an unrelated Rho activator. Thus Daam1 specifically mediates Rho activation by Wnt signaling. Finally, we showed that inhibition of Daam1 function during Xenopus embryogenesis blocks Wnt cell polarity signaling and gastrulation movements but not Wnt/b -catenin signaling or cell fate determination. Our results elucidate a molecular pathway from Wnt/Fz signaling to Rho activation in cell polarity signal transduction. Because Wnt-1 transformation of mouse mammary epithelial cells is always accompanied by morphological changes, which often correlate with Rho GTPase activities, our findings may have potential implications to the understanding of Wnt-1 oncogenic function.

Thus we fully performed and successfully finished the entire Task 2, with the exception of Task 2e (to perform biochemical analysis in mammary cell lines). For both Task 1 and Task 2, we found that injection of Xenopus embryos gave much more profound and

clear-cut results than we were able to obtain in mammary cell lines. One potential reason for this is the difficulty we have encountered with transfecting cDNAs into mammary cells, whose transfection efficiency remained very low despite our repeated efforts. Thus we switched to using several other mammalian cell lines instead (such as human 293T cells, Rat1 cells, etc).

This section contains the highlights of the research results, which represent a large amount of experimental data and methods described in full details in three lengthy and published papers. Thus it is impractical to describe all these results and methods in full details in limited space of this report. I have attached the reprints of these three papers, which are also posted online in these three scientific journals for anyone who is interested.

Conclusions

The Wnt family of secreted growth factors initiates signaling via the Frizzled (Fz) receptor and its co-receptor, LDL receptor-related protein 6 (LRP6), most likely through Wnt-induced Fz-LRP6 complex formation induced by Wnt. Dkk-1 is a high affinity ligand for LRP6, and inhibits Wnt signaling by preventing Fz-LRP6 complex formation that is induced by Wnt. Our findings suggest that Wnt-Fz-LRP6 complex formation, but not Wnt-Fz interaction, triggers Wnt signal transduction.

Dishevelled protein also mediates Wnt/Fz regulation of the Rho GTPase. This function requires a novel Dishevelled-binding protein Daam1. Dishevelled-Daam1-Rho complex formation appears to be critical for Wnt regulation of cell polarity and movements, which are common features for tumor invasion.

In summary, these studies have made some important contributions to the understanding of Wnt signaling in cellular regulation, and may have implications to the mechanism of mammary tumor formation.

Research Accomplishments

The following are the key research accomplishments supported by this award:

1. We demonstrated that members of the mammalian low-density lipoprotein receptor-related protein (LRP) family, LRP5 and 6, function as co-receptors for Fz proteins in Wnt signal transduction to the beta-catenin pathway.

2. We found that the Wnt signaling antagonist Dickkopf-1 (Dkk1) is a high affinity ligand for LRP5/6, and prevents Wnt signaling to beta-catenin via blocking Fz-LRP5/6 complex formation.

3. We showed that Wnt/Fz signaling via the cytoplasmic protein Dishevelled (Dvl) directly activates the RhoA, a small GTPase, in a beta-catenin-independent pathway, and controls cell polarity signal transduction.

4. We identified Daam1 (Dishevelled-associated activator of morphogenesis) via the yeast two-hybrid screen, and found that Daam1 forms a complex with Dvl and RhoA, and mediates Wnt/Fz activation of RhoA.

5. We found that Daam1 is essential for cell movements during Xenopus gastrulation.

Reported Outcomes

The following research manuscripts were generated during the award period:

1. Tamai, K., Semenov, M., Kato, Y., Spokony, R., Liu, C., Katsuyama, Y., Hess, F., Saint-Jeannet, J. P., and He, X. (2000). LDL-receptor-related proteins in Wnt signal transduction. **Nature** 407, 530-535.

2. Semenov, M. V., Tamai, K., Brott, B. K., Kuhl, M., Sokol, S., and He, X (2001). Head inducer Dickkopf-1 is a ligand for Wnt co-receptor LRP6. **Current Biology** 11, 951-961.

3. Habas, R., Kato, Y., and He, X. (2001) Wnt/Frizzled activation of Rho regulates vertebrate gastrulation and requires a Novel Formin Homology Protein Daam1. **Cell** 107, 843-854.

I would like to take this opportunity to acknowledge the support of the DOD Breast Cancer Research Program, which, via this Career Development Award, has made above research projects possible. Through this award, I was able to initiated several high impact and high-risk research efforts in my lab, and was able to focus on science and experiments rather than worrying about financial budgets. I appreciate the generous support from the DOD, and have acknowledged this support in above scientific publications.

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1. Wodarz, A. & Nusse, R. Mechanisms of Wnt signaling in development. Annu. Rev. Cell Dev. Bio. **14**, 59-88 (1998).

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3. Glinka, A., Wu, W., Delius, H., Monaghan, A. P., Blumenstock, C., and Niehrs, C. Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. Nature **391,** 357-362 (1998).

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LDL-receptor-related proteins in Wnt signal transduction

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The Wnt family of secreted signalling molecules are essential in embryo development and tumour formation[1]. The Frizzled (Fz) family of serpentine receptors function as Wnt receptors[2–10], but how Fz proteins transduce signalling is not understood. In *Drosophila*, *arrow* phenocopies the *wingless* (DWnt-1) phenotype[11], and encodes a transmembrane protein[11] that is homologous to two members of the mammalian low-density lipoprotein receptor (LDLR)-related protein (LRP) family, LRP5 and LRP6 (refs 12–15). Here we report that LRP6 functions as a co-receptor for Wnt signal transduction. In *Xenopus* embryos, LRP6 activated

Wnt–Fz signalling, and induced Wnt responsive genes, dorsal axis duplication and neural crest formation. An LRP6 mutant lacking the carboxyl intracellular domain blocked signalling by Wnt or Wnt–Fz, but not by Dishevelled or β-catenin, and inhibited neural crest development. The extracellular domain of LRP6 bound Wnt-1 and associated with Fz in a Wnt-dependent manner. Our results indicate that LRP6 may be a component of the Wnt receptor complex.

Human LRP5 and LRP6 share 71% amino-acid identity[12–15], and together with Arrow, form a distinct subgroup of the LRP family[11]. Arrow, LRP5 and LRP6 each contain an extracellular domain with EGF (epidermal growth factor) repeats and LDLR repeats, followed by a transmembrane region and a cytoplasmic domain lacking recognizable catalytic motifs[11–15]. An lrp6 mutation in mice results in pleiotropic defects recapitulating some, but not all, of the wnt mutant phenotype[16]. To study LRP5/LRP6 involvement in Wnt signalling, we have examined their function in Wnt-induced axis and neural crest formation in *Xenopus* embryos.

Wnt/β-catenin signalling induces dorsal axis formation through activation of responsive genes, including nodal-related 3 (Xnr3) and siamois (sia) (reviewed in ref. 17). Ventral injection of LRP6 RNA into four-cell stage embryos resulted in dorsal axis duplication in a dose-dependent manner (Fig. 1a, b). In animal pole explants, LRP6 induced Xnr3/sia, but not brachyury (Xbra) expression (Fig. 1d), which is activated by mesoderm inducers like activin or basic fibroblast growth factor (bFGF)[17]. These results indicate that overexpression of LRP6 may specifically activate Wnt signalling. To examine whether LRP6 mediates Wnt effect, we co-injected RNAs for LRP6 and Wnt-5a. Neither Wnt-5a nor a low dose of LRP6 alone exhibited any effect, but Wnt-5a plus LRP6 synergistically induced axis duplication and ectopic Xnr3 expression in the embryo (Fig. 1a–c), and activated Xnr3/sia in explants (Fig. 1d). Synergy was also observed between Wnt-5a and hFz5 as reported[4], and between LRP6 and hFz5 (Fig. 1e). Although LRP5 alone did not induce axes, co-injecting LRP5 and Wnt-5a did (Fig. 1a). LDLR alone or in combination with Wnt-5a did not induce axes or Xnr3/sia (Fig. 1a and d). Although Wnt-5a-hFz5 can induce complete axes including head and the notochord[4], Wnt-5a-LRP6 or LRP6 alone (higher doses) induced trunk axis with muscle and neural tissues but lacking head and the notochord (Fig. 1b). This may be explained by quantitative or qualitative differences between Wnt-5a/LRP6 and Wnt-5a/hFz5 co-injections.

Because ectopic Wnt expression enhances, whereas lack of Wnt signalling inhibits, neural crest formation[18–22], we further analysed the effect of LRP6 on neural crest development. LDLR injection had no effect, but LRP6 expression significantly expanded neural crest progenitors in the injected half of the embryo, as determined by the expression of a crest-specific marker, slug (Fig. 2a, b). Thus, overexpression of LRP6 also mimicked Wnt signalling during neural crest formation.

To distinguish whether LRP6 functions in Wnt-responding or Wnt-producing cells, we injected Wnt-5a and LRP6 separately into neighbouring blastomeres at the four-cell stage (Fig. 3a, insert). Induction of secondary axes in embryos and of Xnr3/sia in explants occurred even when Wnt-5a and LRP6 were expressed in different cells (Fig. 3a, b). Therefore, LRP6 is probably involved in respond-

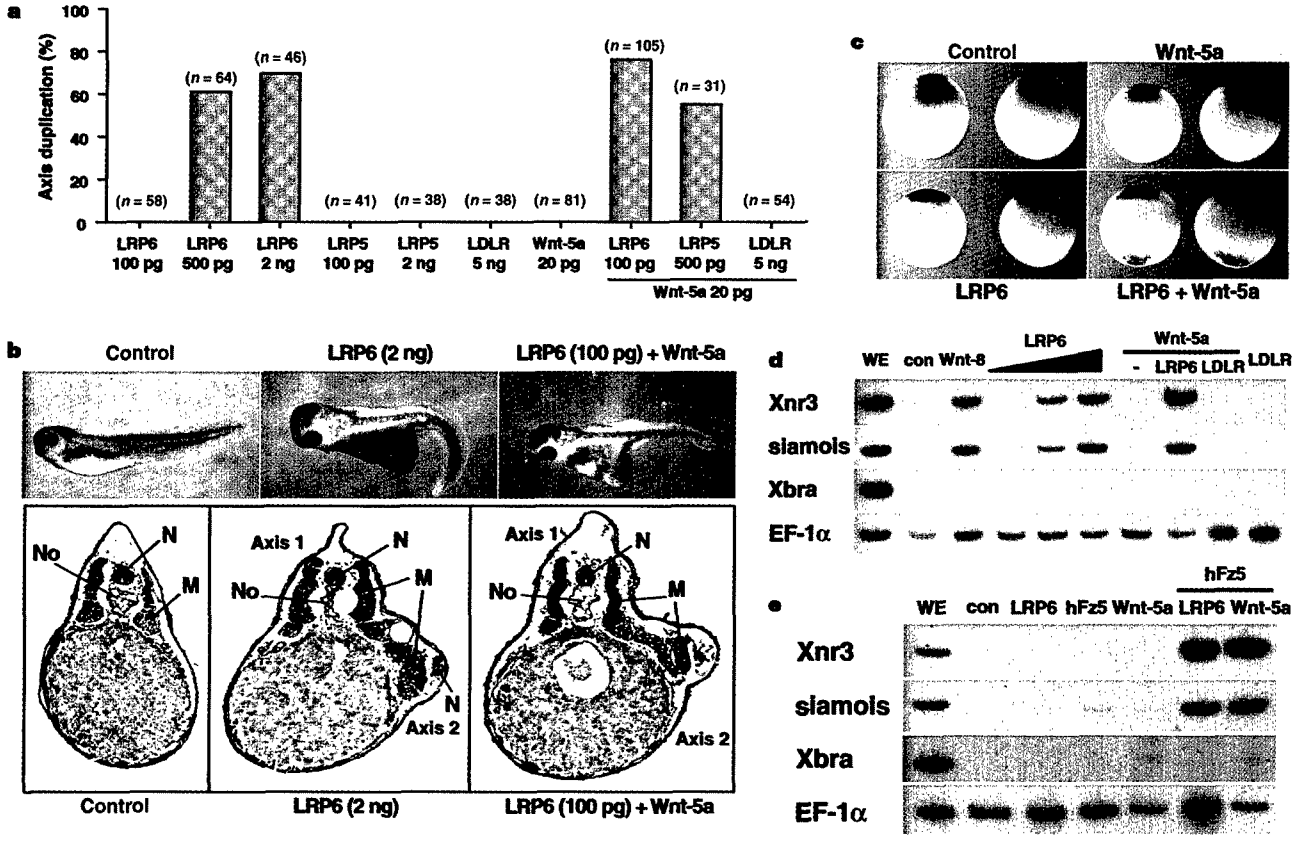

Figure 1 LRP6/LRP5 in Wnt signalling. **a**, Ventral injection of LRP6 RNA (0.5 or 2 ng), or co-injection of RNAs for *Xenopus* Wnt-5a (20 pg) plus LRP6 (100 pg) or LRP5 (500 pg) induced axis duplication. LDLR RNA (5 ng or 1 ng; not shown) alone or co-injected with Wnt-5a did not induce axes. *n*, numbers of embryos scored. **b**, LRP6 (2 ng) or Wnt-5a (20 pg) plus LRP6 (100 pg) induced trunk duplication lacking head and the notochord. Top, stage-40 embryos; bottom, histology on cross-sections. M, muscle; N, neural tissues; No, notochord. **c**, Ventral co-injection of Wnt-5a (20 pg) and LRP6 (100 pg) activated ectopic Xnr3 expression (stage 10.5), assayed by *in situ* hybridization ($n = 10–12$). **d**, **e**, Synergistic induction of Xnr3/sia in animal pole explants (stage 10.5) by LRP6 (100 pg) plus Wnt-5a (20 pg) or hFz5 (400 pg), assayed by RT–PCR. The RNA amount injected is as in **a** except for *Xenopus* Wnt-8 (10 pg). EF-1α, loading control. WE, whole embryos (stage 10.5); con, explants from uninjected embryos.

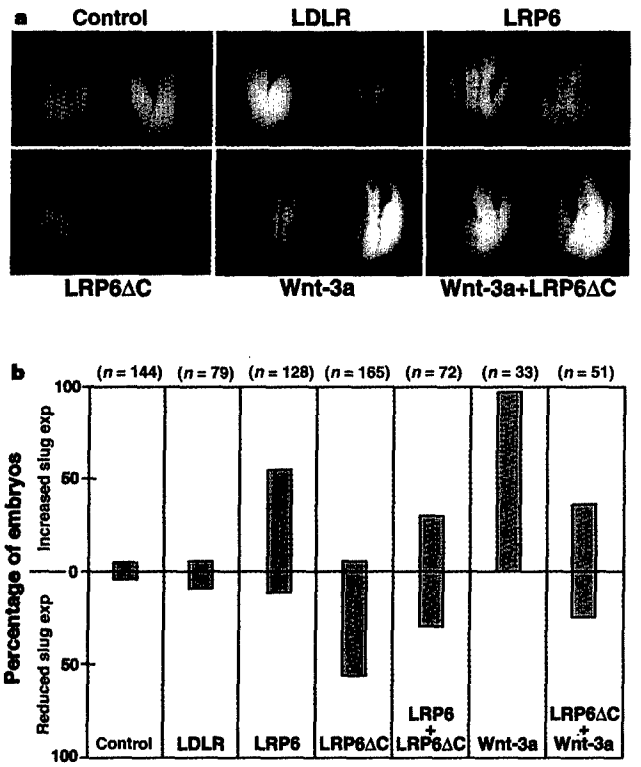

Figure 2 LRP6 in neural crest formation. **a**, Slug expression (stage 15–20) examined by *in situ* hybridization. LRP6 RNA (2 ng) or *Xenopus* Wnt-3a DNA (100 pg) increased, whereas LRP6ΔC (2 ng) inhibited slug expression in the injected half (the left side, labelled by red-coloured staining derived from co-injected β-galactosidase RNA). LRP6ΔC antagonized Wnt-3a, whose DNA was used to mimic zygotic Wnt expression. LDLR (2 ng) had no effect. **b**, Summary of the *in situ* hybridization results.

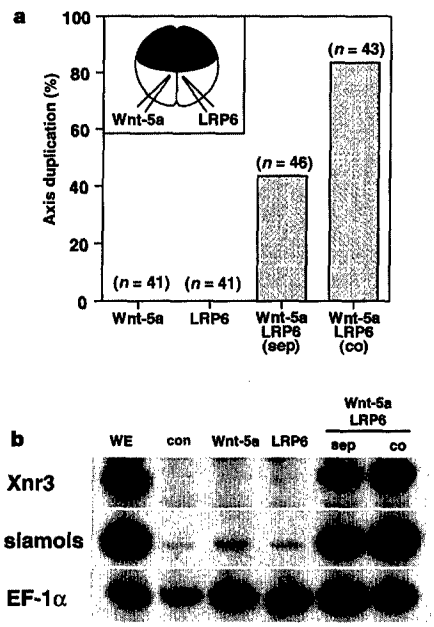

Figure 3 LRP6 functions in Wnt-responding cells. RNAs for Wnt-5a (100 pg) and LRP6 (100 pg) were separately injected (**a**, insert) into two neighbouring blastomeres in the ventral equatorial region (**a**) or in the animal pole (**b**). sep, separate injection; co, Wnt-5a and LRP6 co-injected into a single blastomere for comparison. **a**, Percentage of embryos with axis duplication. **b**, Xnr3/sia induction in animal pole explants. Separate injections were less effective than co-injections (**a**, **b**), probably because of limited diffusion of Wnt-5a protein. EF-1α, loading control. WE, whole embryos (stage 10.5); con, explants from uninjected embryos.

ing to, rather than in enhancing, the production or secretion of the Wnt ligand.

To inhibit the function of the endogenous LRP6 which is expressed maternally and throughout embryogenesis (Fig. 4f), we generated LRP6ΔC which has most of its cytoplasmic domain deleted. LRP6ΔC did not, either alone or in combination with Wnt-5a, induce axes or activate Xnr3/sia (Fig. 4a, b), but inhibited axis duplication and Xnr3/sia induction by the wild-type LRP6 (Fig. 4a, b). This inhibition was counteracted by an increasing amount of co-injected LRP6 (Fig. 4a). These data suggest that LRP6ΔC is a dominant interfering mutant for LRP6 or related molecules, and that LRP6 cytoplasmic domain is required for Wnt signalling. LRP6ΔC inhibited Xnr3/sia induction by several Wnt molecules, including Wnt-1, Wnt-2, Wnt-3a and Wnt-8 (Fig. 4c). LRP6ΔC also inhibited Wnt-5a signalling through hFz5 (Fig. 4c), showing that hFz5, and probably other endogenous Fz molecules mediating Wnt-1 or Wnt-8 signalling, depend on LRP6 or related proteins. LRP6ΔC did not affect Xbra induction by activin or bFGF (Fig. 4d), and thus interferes specifically with Wnt signalling.

LRP6ΔC injected dorsally at the four-cell stage did not perturb the endogenous axis formation (data not shown). This may mean that the dorsal β-catenin pathway is activated by mechanisms other than Wnt stimulation[17]; alternatively, the dorsal Wnt–Fz signalling may occur early[23] before LRP6ΔC can interfere. However, LRP6ΔC inhibited neural crest development as examined by slug expression, and suppressed ectopic crest formation induced by Wnt-3a DNA (Fig. 2a and b). Furthermore, co-injection of LRP6 rescued LRP6ΔC inhibition of crest formation (Fig. 2b). Thus, LRP6 or a related molecule is required for Wnt-dependent neural crest formation *in vivo*.

In the current model of Wnt/β-catenin signalling, Wnt stimulation of a Fz receptor activates the intracellular protein Dishevelled (Dsh or Dvl), thereby antagonizing the inhibitory action of the Axin/GSK-3 complex and stabilizing β-catenin, which together

with the transcription factor TCF/LEF activates responsive genes[1]. To position LRP6 in this cascade, we tested relationships between LRP6 and other Wnt signalling components. LRP6ΔC inhibited Xnr3/sia induction by Wnts, but not by Dsh or β-catenin (Fig. 4c), suggesting that LRP6ΔC interfered with Wnt signalling upstream of Dsh function. Supporting this epistasis, LRP6 induction of Xnr3/sia was antagonized by Axin[24], by a dominant-negative TCF, ΔNTCF[25], and by a dominant-negative Dsh, mDvl2-DIX (Fig. 4e). LRP6 activity was also inhibited by frizzled-related protein (FRP), a secreted Wnt antagonist[26] (Fig. 4e), implying that LRP6 activation of Wnt signalling relied on endogenous Wnt molecules. Alternatively, FRP may directly inhibit LRP6. Thus, LRP6 acts between the extracellular Wnt, FRP and intracellular Dsh, possibly as a co-receptor for Wnt molecules.

To function as a Wnt co-receptor, LRP6 should bind Wnt or Fz or both. To examine the issue, we used a secreted form of mFz8, mFz8CRD–IgG (ref. 8), which comprised the cysteine-rich domain (CRD) of mFz8 N-terminal extracellular region fused with the immunoglobulin-γ (IgG) Fc epitope, and a secreted LRP6N–Myc, which consisted of the LRP6 extracellular domain tagged by the Myc epitope. We incubated mFz8CRD–IgG and LRP6N–Myc proteins with or without Wnt-1. mFz8CRD–IgG co-precipitated LRP6N–Myc only in the presence of Wnt-1; the secreted IgG fusion partner failed to do so regardless of Wnt-1 (Fig. 5A). We also performed a reciprocal precipitation using secreted LRP6N–IgG and mFz8CRD–Myc, which we generated by swapping the two epitopes. LRP6N–IgG co-precipitated mFz8CRD–Myc, again only in the presence of Wnt-1, whereas the control IgG did not (Fig. 5B). LRP6N–IgG also co-precipitated Wnt-1–Myc (Fig. 5C), a tagged Wnt-1 protein. These results suggest that the extracellular domain of LRP6 can bind Wnt-1 and form a complex with Fz in a Wnt-dependent fashion.

We have shown that in two developmental processes dependent on the Wnt pathway in *Xenopus*—secondary axis and neural crest formation—LRP6 activates but a dominant-negative LRP6 inhibits Wnt signalling, providing compelling evidence that LRP6 is critical in Wnt signal transduction. LRP6 functions upstream of Dsh in Wnt-responding cells, synergizes with either Wnt or Fz, and importantly, is able to bind Wnt-1 and to associate with Fz in a Wnt-dependent manner. The simplest interpretation of these findings is that LRP6 is a component of the Wnt–Fz receptor complex. Genetic studies of *arrow* in *Drosophila*[11] and lrp6 in mice[16] strongly support this hypothesis. Our binding data also raise the possibility that Wnt-induced formation of the Fz–LRP6 complex assembles LRP6, Fz and their associated proteins, thereby initiating cytoplasmic signalling. Consistent with this notion, Wnt signal transduction requires intracellular regions of both Fz (data not shown) and LRP6, which harbours candidate protein–protein interaction motifs[11–15]. Notably, *arrow* does not exhibit *fz* planar polarity phenotype[11], implying that Arrow–LRP6 may specify Wnt–Fz signalling towards

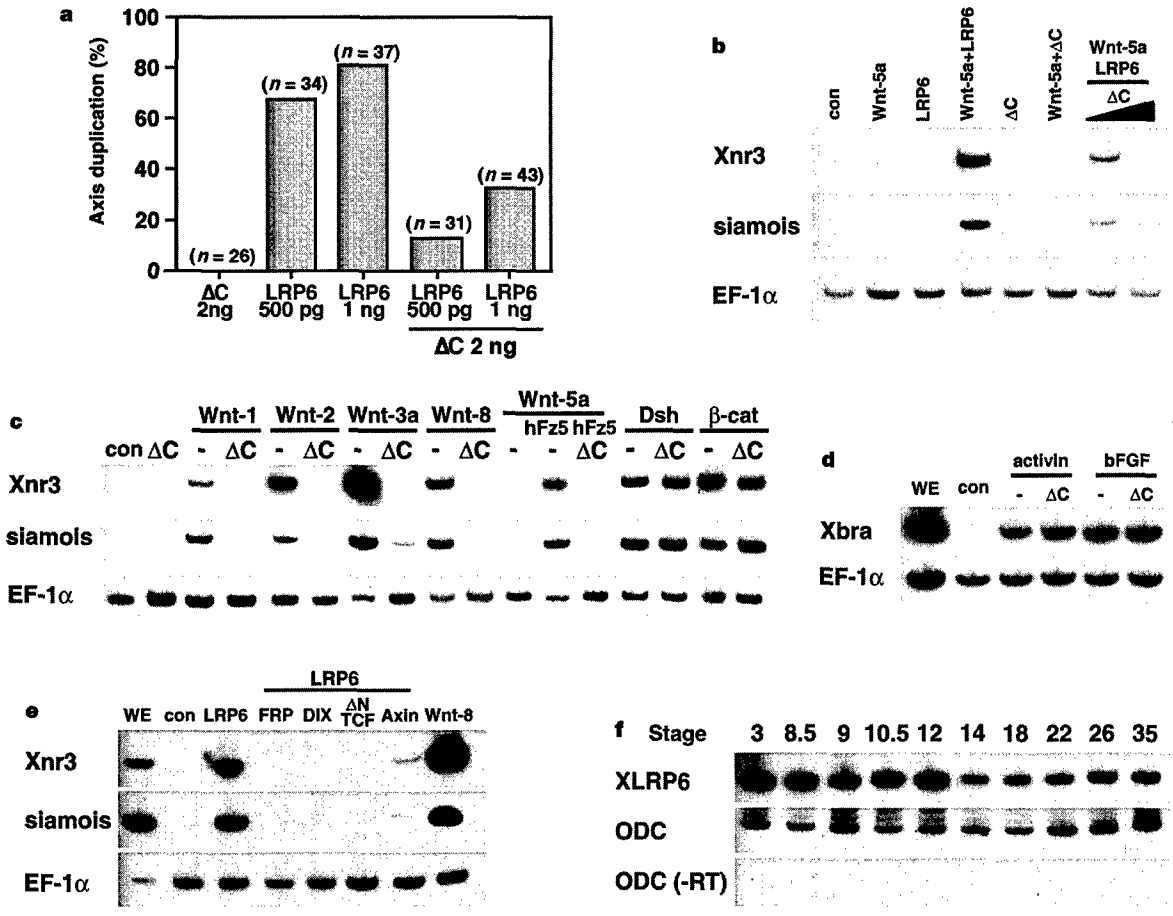

Figure 4 LRP6 functions upstream of Dsh protein and is antagonized by FRP. **a**, LRP6ΔC (labelled ΔC, 2 ng) did not induce axes but inhibited axis induction by LRP6 (500 pg) when co-injected. An increase of LRP6 RNA (1 ng) counteracted LRP6ΔC inhibition. **b**–**e**, Animal pole explant assays. **b**, LRP6ΔC alone (2 ng) or Wnt-5a (20 pg) plus LRP6ΔC (100 pg) did not induce Xnr3/sia. LRP6ΔC (500 pg and 2 ng) inhibited Xnr3/sia induced by Wnt-5a (20 pg) plus LRP6 (100 pg) in a dose dependent manner. **c**, LRP6ΔC (2 ng) inhibited Xnr3/sia induction by Wnt-1 (10 pg), Wnt-2 (40 pg), Wnt-3a (5 pg), Wnt-8 (10 pg) and by Wnt-5a (20 pg) plus hFz5 (100 pg), but not by Dsh (1 ng) or β-catenin (100 pg). *Xenopus* Wnts were used except for mouse Wnt-1. **d**, LRP6ΔC (2 ng) did not perturb Xbra induction by activin or bFGF (50 ng ml^{-1}). **e**, LRP6 (500 pg) induction of Xnr3/sia was inhibited by co-injected RNAs (2 ng) for FRP, a dominant-negative Dsh (DIX), ΔNTCF and Axin. **f**, *Xenopus* LRP6 is expressed maternally and throughout embryogenesis, as assayed by RT–PCR. Ornithine decarboxylase was used as a control for relative RNA amount. –RT, PCR without RT. A partial cDNA of XLRP6 showed 80% amino-acid sequence identity with the corresponding region of human LRP6. EF-1α, loading control. WE, whole embryos (stage 10.5); con, explants from uninjected embryos.

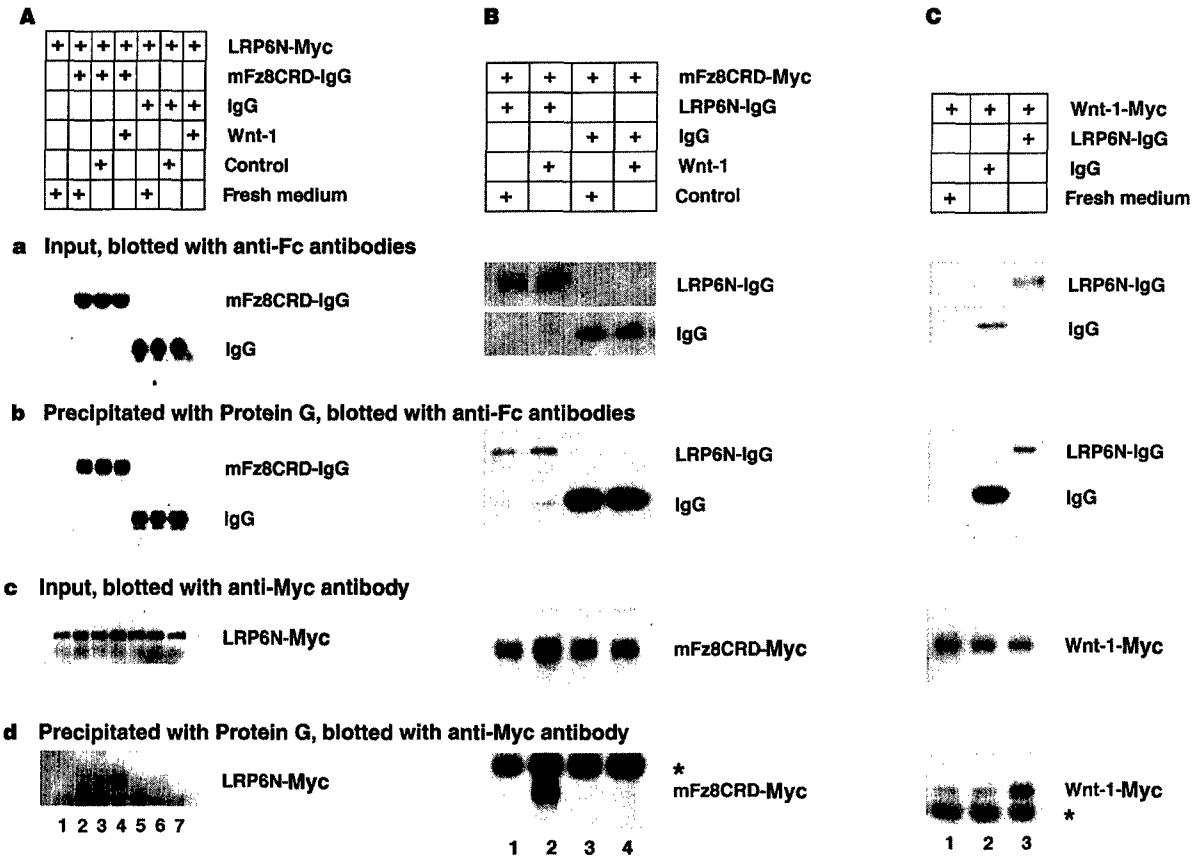

Figure 5 LRP6 extracellular domain binds Wnt-1 and complexes with mFz8CRD in the presence of Wnt-1. All components were provided as conditioned medium. **A**, mFz8CRD–IgG co-precipitated LRP6N–Myc in the presence of Wnt-1. Conditioned medium mixtures containing mFz8CRD–IgG or the control IgG (**a**) and LRP6N–Myc (**c**) were incubated in the presence of Wnt-1 conditioned medium, control conditioned medium, or fresh medium, and precipitated with Protein G beads. Precipitates were detected for mFz8CRD–IgG or the control IgG (**b**) or LRP6N–Myc (**d**). **B**, LRP6N–IgG co-precipitated mFz8CRD–Myc in the presence of Wnt-1. Note that the epitope tags were reciprocated. **C**, LRP6N–IgG co-precipitated Wnt-1–Myc. Precipitation of LRP6N–IgG was less effective than that of IgG in (**B**, **C**). Asterisk indicates IgG from bovine serum.

the β-catenin pathway. How Fz, LRP6 and proteoglycan molecules such as Dally[27] interact to mediate Wnt recognition/specificity and signal transduction remains to be studied. In addition, whether other LRPs and LRP-binding proteins participate in or modulate different Wnt–Fz signalling pathways needs evaluation. The LRP family have diverse signalling functions, as shown here[11,16] and in mammalian brain development[28]. □

Methods

cDNA constructs

We subcloned cDNAs for human LRP5 (ref. 13), LRP6 (ref. 12) and LDLR into pCS2+. LRP6ΔC was generated by deleting residue 1,440 onwards, 3′ of an internal *Xho*I site. mFz8CRD–IgG and the control human IgG (Fc) have been described[8]. LRP6N–IgG and LRP6N–Myc were generated by fusing the LRP6 extracellular domain (two residues before the putative transmembrane region) with the IgG[8] or the 6–Myc epitope (from pCS2+MT). mFz8CRD–Myc and Wnt-1–Myc comprised residues 1–178 of mFz8, and mouse Wnt-1 fused with the 6–Myc epitope, respectively. We generated mDvl2–DIX by subcloning the *Nco*I and *Xmn*I fragment (encoding the DIX domain) of murine Dvl2 cDNA into pCS2+MT. mDvl2-DIX behaves as a dominant-negative reagent that blocks Wnt signalling. Details of the plasmids are available upon request.

Embryo manipulation, histology, *in situ* hybridization and explants

These procedures, and polymerase chain reaction with reverse transcription (RT–PCR), were done as described[19,29].

Conditioned medium, co-precipitation and immunoblotting

We produced LRP6N–IgG (apparent relative molecular mass 200K), mFz8CRD–IgG (60K), the control IgG (35K), LRP6N–Myc (200K) and mFz8CRD–Myc (45K) by transient transfection of 293T cells as described[8]. Wnt-1 or Wnt-1–Myc (70K) conditioned medium was collected from Rat-2 cells infected with viral vectors (ref. 30, and data not shown). Wnt-1 or Wnt-1–Myc biological activity was controlled using a β-catenin stabilization assay (data not shown). LRP6N–IgG (1 ml) or mFz8CRD–IgG (0.5 ml), or a corresponding amount of the control IgG was mixed with 0.5 ml of mFz8CRD–Myc or LRP6N–Myc in the presence of 2 ml of Wnt-1, control, or fresh medium. Precipitation and immunoblotting were done similarly to ref. 8.

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An LDL-receptor-related protein mediates Wnt signalling in mice

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Wnt genes comprise a large family of secreted polypeptides that are expressed in spatially and tissue-restricted patterns during vertebrate embryonic development[1]. Mutational analysis in mice has shown the importance of Wnts in controlling diverse developmental processes such as patterning of the body axis, central nervous system and limbs, and the regulation of inductive events during organogenesis[2]. Although many components of the Wnt signalling pathway have been identified, little is known about how Wnts and their cognate Frizzled receptors signal to downstream effector molecules. Here we present evidence that a new member of the low-density lipoprotein (LDL)-receptor-related protein family, LRP6 (ref. 3), is critical for Wnt signalling in mice. Embryos homozygous for an insertion mutation in the LRP6 gene exhibit developmental defects that are a striking composite of those caused by mutations in individual Wnt genes. Furthermore, we show a genetic enhancement of a Wnt mutant phenotype in mice lacking one functional copy of LRP6. Together, our results support a broad role for LRP6 in the transduction of several Wnt signals in mammals.

In a screen for recessive lethal phenotypes in mice caused by gene trap insertions in cell-surface proteins[4], we recovered an insertion mutation that joined the first 321 amino acids of the LRP6 protein in-frame with the *βgeo* reporter gene. Embryos homozygous for the insertion in LRP6 died at birth, and exhibited a variety of severe developmental abnormalities including a truncation of the axial skeleton, limb defects, microophthalmia and malformation of the urogenital system (Fig. 1a–c). Northern blot analysis showed a complete absence of LRP6 transcripts in $LRP6^{-/-}$ embryos and embryonic fibroblasts (Fig. 1d). Southern blot analysis indicated a simple insertion event in which LRP6 exons downstream of the vector are retained (data not shown). On the basis of *βgeo* reporter activity, which accurately reports endogenous gene expression[4], the LRP6 gene appears to be expressed in all cells of the developing embryo (data not shown).

The specific developmental defects that we observed in homozygous embryos were remarkably similar to mice carrying mutations in Wnt genes, specifically Wnt-3a, Wnt-1 and Wnt-7a. Both the targeted null mutation and a classical hypomorphic allele of Wnt-3a, *vestigial tail* (*vt*), cause caudal truncations of the body axis[5–7]. We observed a similar axial truncation in $LRP6^{-/-}$ neonates in which vertebrae caudal to the lumbar regions are absent (Fig. 1b). This was first evident as a reduction in the size of the tailbud at 8.5 days post coitum (d.p.c.), with the loss of paraxial mesoderm and caudal somites at later stages (Fig. 2a, b). Sections through the tailbud of $LRP6^{-/-}$ embryos revealed an excess of neural tissue and a corresponding loss of paraxial mesoderm (Fig. 2c, d), similar to what was observed in the targeted Wnt-3a mutant[6]. As in *vt*

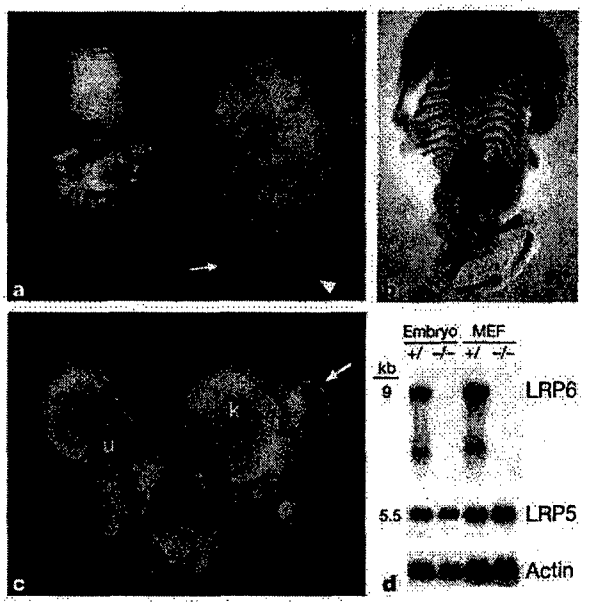

Figure 1 LRP6 mutant embryos exhibit severe developmental defects **a**, Embryo at 15.5 d.p.c. showing spina bifida (arrowhead), absence of a tail, malformed fore and hindlimbs (arrow) and retinal coloboma. **b**, Skeleton of a neonate showing truncation of the axial skeleton and loss of distal limb structures. **c**, Urogenital system of an 18.5-d.p.c. female embryo with small, cystic kidneys (k), expanded ureter (u) and developmentally delayed ovaries (arrow). **d**, Northern blot of total RNA from non-mutant (+/) and mutant (−/−) 12.5-d.p.c. embryos and embryonic fibroblasts (MEF).

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the dorsal leg exhibit no such defects (data not shown). This result also shows that the Fz receptor is available to respond to tissue polarity signals in the absence of *arrow* function and one would therefore expect it to be at the cell surface in *arrow* mutant cells. This finding suggests that Arrow does not merely act as a chaperone to guide Fz-class proteins to the cell surface. This again is consistent with our model that Fz and Arrow have a similar role in the reception of the Wg signal and that they may both be part of a single receptor complex. Recent work raises the possibility that other LRPs are part of a signal reception complex, just as we postulate for Arrow. The VLDL and ApoE receptors (both LRPs) are essential during mouse cerebellar development, where they bind the ligand, Reelin, while intracellularly binding to, and inducing the phosphorylation of, the adapter protein, Disabled-1 (reviewed in ref. 25). In addition to the LRPs, a second receptor—the Cadherin-related neuronal receptor—also binds Reelin, while its intracellular domain associates with the Fyn tyrosine kinase. This suggests that as the two receptor subunits bind Reelin, two proteins are brought into proximity inside the cell, Disabled-1 and Fyn[26]. Perhaps Arrow and the Fz proteins similarly bind ligand and consequently bring together proteins in the cytoplasm that initiate Wg/Wnt signalling. □

Methods

Two cDNAs, corresponding to CT15575, isolated from a 0-4h library[27] contained an open reading frame preceded by stop codons in all frames. Two ATGs are found 5′ to a putative signal peptide. For UAS–Arrow, cDNA F1 from pNB40 was cloned into pUASg as a *XhoI*/ partial *NotI* fragment.

arr^{2} germline clones[28] were induced in HS-Flp/+; FRT42B *arr*^{2}/FRT42B *ovo*^{D1} females mated to Df(2R)8-104/CyO or Df(2R)AA1/CyO Hb-lacZ males, and null embryos were identified by absence of Hb-lacZ staining. Disc clones were induced in *y w* hs-Flp; *dpp*^{10638} (or Dll–LacZ) FRT43D *arr*^{2}/FRT43D pi-Myc (45F, 47F) larvae[29]. Confocal projections were captured with LSM510 software on a Zeiss axiovert microscope. For adult wings, MS1096–GAL4 was used to express UAS–DFz2 (ref. 19), or UAS-FRT-white+FRT-Arrow. Flies analysed were of genotype *y w* hs-flipase MS1096/ *y w*; UAS-DFz2/+ *y w* hs-flipase MS1096/ *w*; Df(2)8-104 UAS-FRT white+ FRT-Arrow /+, where the white+FRT cassette was removed in clones after heat-shock-induced flipase recombination[22]. Marked *arr* clones were induced while overexpressing DFz2 in *y w* hs-flipase MS1096/ *y w*; FRT42D *arr*^{2}/ FRT42D y+ w+; UAS-DFz2/+ flies.

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LDL-receptor-related proteins in Wnt signal transduction

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The Wnt family of secreted signalling molecules are essential in embryo development and tumour formation[1]. The Frizzled (Fz) family of serpentine receptors function as Wnt receptors[2–10], but how Fz proteins transduce signalling is not understood. In *Drosophila*, *arrow* phenocopies the *wingless* (DWnt-1) phenotype[11], and encodes a transmembrane protein[11] that is homologous to two members of the mammalian low-density lipoprotein receptor (LDLR)-related protein (LRP) family, LRP5 and LRP6 (refs 12–15). Here we report that LRP6 functions as a co-receptor for Wnt signal transduction. In *Xenopus* embryos, LRP6 activated

Head inducer Dickkopf-1 is a ligand for Wnt coreceptor LRP6

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Background: Dickkopf-1 (Dkk-1) is a head inducer secreted from the vertebrate head organizer and induces anterior development by antagonizing Wnt signaling. Although several families of secreted antagonists have been shown to inhibit Wnt signal transduction by binding to Wnt, the molecular mechanism of Dkk-1 action is unknown. The Wnt family of secreted growth factors initiates signaling via the Frizzled (Fz) receptor and its candidate coreceptor, LDL receptor-related protein 6 (LRP6), presumably through Fz-LRP6 complex formation induced by Wnt. The significance of the Fz-LRP6 complex in signal transduction remains to be established.

Results: We report that Dkk-1 is a high-affinity ligand for LRP6 and inhibits Wnt signaling by preventing Fz-LRP6 complex formation induced by Wnt. Dkk-1 binds neither Wnt nor Fz, nor does it affect Wnt-Fz interaction. Dkk-1 function in head induction and Wnt signaling inhibition strictly correlates with its ability to bind LRP6 and to disrupt the Fz-LRP6 association. LRP6 function and Dkk-1 inhibition appear to be specific for the Wnt/Fz β-catenin pathway.

Conclusions: Our results demonstrate that Dkk-1 is an LRP6 ligand and inhibits Wnt signaling by blocking Wnt-induced Fz-LRP6 complex formation. Our findings thus reveal a novel mechanism for Wnt signal modulation. LRP6 is a Wnt coreceptor that appears to specify Wnt/Fz signaling to the β-catenin pathway, and Dkk-1, distinct from Wnt binding antagonists, may be a specific inhibitor for Wnt/β-catenin signaling. Our findings suggest that Wnt-Fz-LRP6 complex formation, but not Wnt-Fz interaction, triggers Wnt/β-catenin signaling.

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Background

Spemann's organizer, which is located at the upper dorsal blastopore lip in amphibian embryos at the beginning of gastrulation, is essential for the basic body plan [1]. Among many of its critical functions, the organizer induces head and trunk formation via distinct head and trunk organizers [2–4]. The formation and function of Spemann's organizer depend on signal transduction mediated by the Wnt family of secreted growth factors [1, 5, 6]. Activation of the maternal Wnt/β-catenin pathway induces the formation of Spemann's organizer, which, upon the start of zygotic transcription, expresses many secreted signaling molecules with head- or trunk-inducing activities [1, 7, 8]. Many of the inducers belong to two functional classes that antagonize signaling by either Wnt or BMP (bone morphogenetic protein; [1, 5, 7]). Recent experiments suggest that trunk development can be achieved by the inhibition of BMP signaling, whereas head formation is induced via simultaneous inhibition of both BMP and Wnt signaling [9]. Indeed, head inducers such as Cerberus, Dickkopf-1 (Dkk-1), Frzb, and Crescent are Wnt antagonists (Cerberus also antagonizes BMP and Nodal signaling) and are expressed in regions that are critical for anterior patterning [10–17]. Although these Wnt antagonists represent distinct polypeptide families, Cerberus, Frzb, and another antagonist, WIF-1, have been shown to bind Wnt proteins and presumably prevent Wnt from binding to receptors [11, 12, 14, 18]. Dkk-1 represents a novel multigene family that is found throughout vertebrate species, including humans [13, 19–23], and is essential for *Xenopus* head formation [13]. How Dkk-1 inhibits Wnt signal transduction to induce head formation is unknown.

Wnt signaling is mediated by the Frizzled (Fz) family of seven-pass transmembrane receptors that bind Wnt via the conserved amino-terminal cysteine-rich domain (CRD) [24]. In fact, Frzb/Crescent proteins share a homologous CRD and thereby compete with Fz receptors for Wnt [11, 12, 25–28]. Recently, a single-pass transmembrane receptor, LDL (low-density lipoprotein) receptor-related protein 6 (LRP6), was shown to be essential for Wnt signaling in *Drosophila* [29], *Xenopus* [30], and mice [31]. LRP6 synergizes with Fz in Wnt signaling and, intriguingly, is able to bind Wnt-1 and to associate with Fz8CRD in a Wnt-dependent fashion [30]. This finding suggests

that LRP6 is a component of the Wnt receptor complex. The significance of the Fz-LRP6 complex in Wnt signaling remains to be established.

Here we report that, in contrast to characterized Wnt binding antagonists, Dkk-1 is a high-affinity ligand for LRP6 and inhibits Wnt signaling by binding to LRP6 and preventing Wnt-induced Fz-LRP6 complex formation. Dkk-1 neither possesses significant Wnt binding activity nor interferes with Wnt-Fz interaction, and Dkk-1 activity inducing head formation and antagonizing Wnt signaling strictly correlates with its ability to bind LRP6 and to interrupt the Fz-LRP6 association. LRP6 function and Dkk-1 inhibition appear to be specific for the Wnt/β-catenin signaling pathway. These results reveal the molecular interplay between Dkk-1 and the Fz-LRP6 complex in head induction, Wnt signal transduction, and Wnt pathway specificity.

Results

Dkk-1 inhibits Wnt signaling without binding to Wnt-1 or interfering with Wnt-Fz interaction

RNA coinjection experiments showed that Dkk-1 antagonizes Wnt signaling upstream of Dishevelled [13]. This finding suggests that Dkk-1 may, like other Wnt antagonists, act to inhibit Wnt signal reception. We compared Dkk-1 with mFz8CRD [32], which inhibits Wnt signaling by binding the Wnt ligand in a manner analogous to that of Frzb. Cotransfection of cDNAs for human Dkk-1 or mFz8CRD with Wnt-1 inhibited β-catenin induction by Wnt-1 (not shown). To rule out the possibility that Dkk-1 inhibits Wnt signaling by interfering with Wnt-1 production or secretion, we examined whether secreted Dkk-1 protein could inhibit Wnt-1 protein after secretion. Wnt-1-conditioned medium (CM) increased cytosolic β-catenin protein; this increase was suppressed by CM containing Dkk-1-Flag (tagged with the Flag epitope; Figure 1a) and by CM containing mFz8CRD-IgG (tagged with the immunoglobulin-γ Fc epitope [32]). The inhibitory activity toward Wnt-1 was due to Dkk-1 protein in the CM, as confirmed by immunodepletion with Protein G beads (not shown). These results demonstrated that secreted Dkk-1 protein directly antagonizes Wnt-1 signaling in the extracellular space.

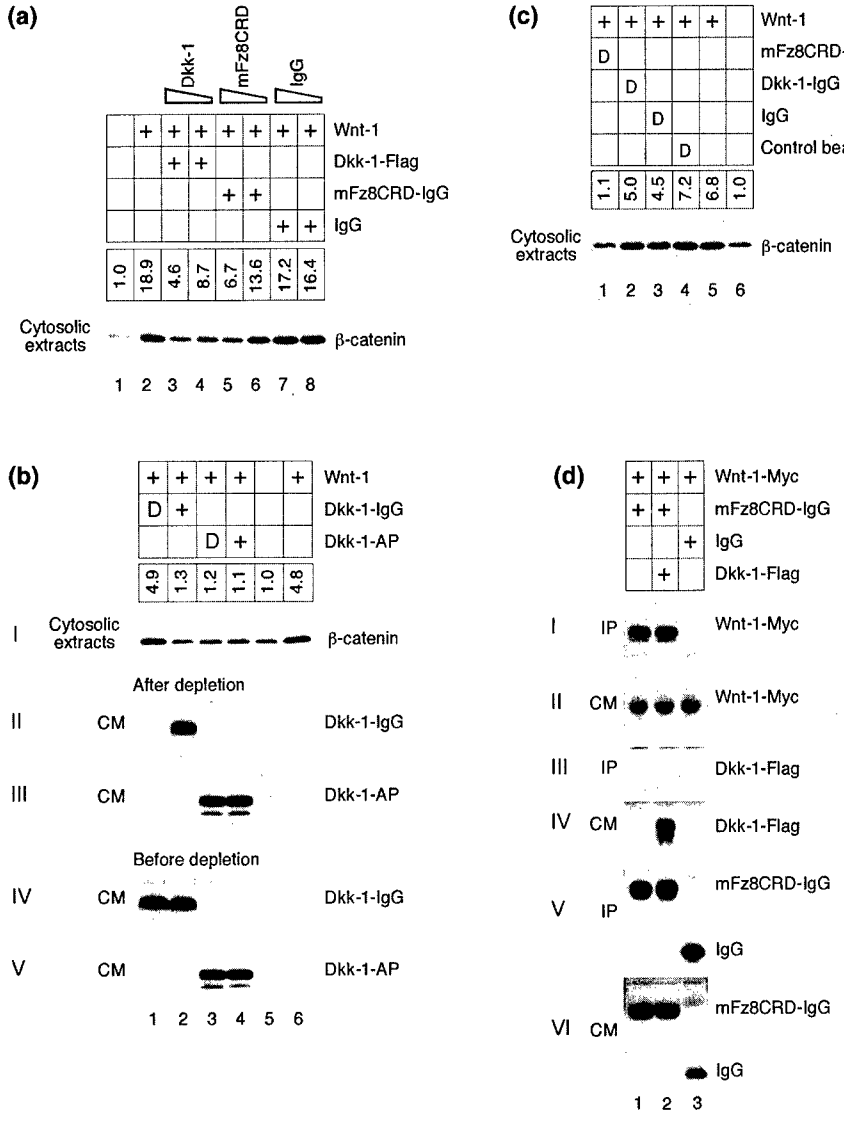
If inhibition of Wnt signaling by secreted Dkk-1 was a result of Dkk-1 binding to Wnt, we expected to remove Wnt-1 activity from Wnt-1 CM by using Dkk-1 as an affinity depletion reagent. We mixed Wnt-1 CM with Dkk-1-IgG CM, then depleted Dkk-1-IgG via Protein G beads (Figure 1b). Depletion of Dkk-1-IgG did not remove Wnt-1, as evidenced by the full restoration of Wnt induction of β-catenin after removal of Dkk-1-IgG (Figure 1b). In control experiments, the same Protein G depletion procedure was not able to remove Dkk-1-AP (tagged with the alkaline phosphatase) from the CM mixture of Wnt-1 plus Dkk-1-AP, and consequently, Wnt-1 activity was still suppressed (Figure 1b). We compared Dkk-1 with mFz8CRD as affinity depletion reagents. We incubated Wnt-1 CM with Protein G beads that were preloaded with the control IgG or a large amount of either Dkk-1-IgG or mFz8CRD-IgG (equivalent to 50 times the amount required for inhibition of Wnt-1 CM in the β-catenin stabilization assay). mFz8CRD-IgG-loaded Protein G beads completely depleted Wnt-1 activity, whereas Dkk-1-IgG-loaded Protein G beads failed to do so (Figure 1c). Therefore, Dkk-1 has no significant Wnt binding capacity and appears to inhibit Wnt-1 by a mechanism distinct from that of mFz8CRD. The fact that removal of Dkk-1 fully restores Wnt-1 activity also argues against the possibility that Dkk-1 antagonizes Wnt-1 by enzymatic modification or inactivation of Wnt-1.

Dkk-1 may bind to Fz and prevent Wnt-Fz interaction, or it may bind to and inactivate the Wnt-Fz complex. In fact, FRP/sFRP-1, a member of the Frzb family of Wnt antagonists, has been shown to heterodimerize with a FzCRD [33]. To examine these possibilities, we incubated CM containing Wnt-1-Myc (with the Myc epitope) and mFz8CRD-IgG in the presence or absence of Dkk-1-Flag. mFz8CRD did not precipitate any detectable amount of Dkk-1 regardless of the presence of Wnt-1 (Figures 1d and 2a) and bound to a similar amount of Wnt-1 regardless of the presence of Dkk-1 (Figure 1d). Therefore, Dkk-1 binds neither mFz8CRD nor the Wnt-1-mFz8CRD complex, nor does Dkk-1 interfere with Wnt-1-mFz8CRD complex formation.

Dkk-1 is a ligand for LRP6 and disrupts Fz-LRP6 complex formation induced by Wnt-1

Recent studies demonstrated that LRP6 is required for Wnt signaling [29–31], possibly as a component of the Wnt receptor complex. We examined whether Dkk-1 interacts with LRP6. Strikingly, the LRP6 extracellular domain, LRP6N-IgG, coprecipitated Dkk-1-Flag when the two CM were combined (Figure 2a), whereas neither mFz8CRD-IgG nor the control IgG, each of which was present at a much higher protein level, precipitated Dkk-1 (Figure 2a). Importantly, LDLRN-IgG, the extracellular domain of the related LDL receptor (LDLR) that is not involved in Wnt signaling [30], did not precipitate Dkk-1 despite its higher abundance as compared to LRP6N (Figure 2a). Conversely, Dkk-1-IgG coprecipitated LRP6N-Myc, but not LDLRN-Myc, when CM for each protein were mixed (Figure 2b). In fact, Dkk-1 failed to bind to LDLRN even when a 20-fold molar excess of LDLRN was present (Figure 2b). This result again demonstrated the high selectivity of Dkk-1-LRP6 interaction. Using Dkk-1-AP and LRP6N-IgG in a liquid-phase, enzyme-linked binding assay, we demonstrated that Dkk-1-LRP6 interaction was specific and saturable and exhibited an affinity constant (Kd) of 0.5 nM, as determined with binary binding Scatchard analysis (Figure 2c).

Figure 1



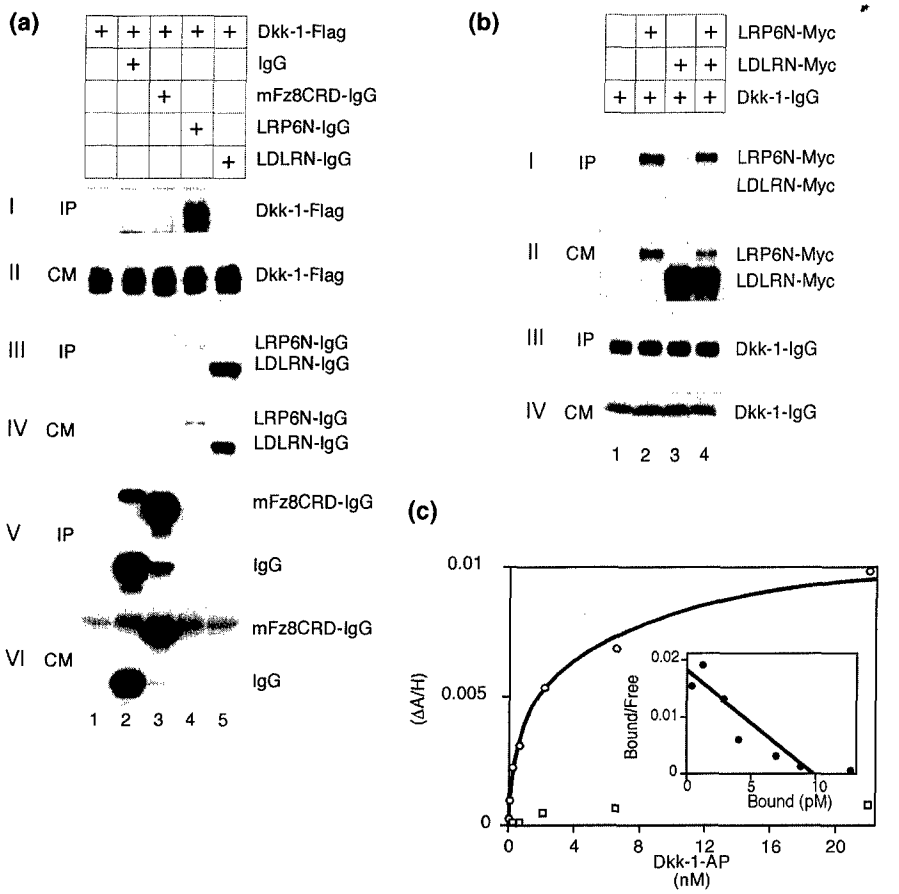
Dkk-1 inhibits Wnt-1 without binding to Wnt-1, Fz, or the Wnt-Fz complex. **(a)** Dkk-1 CM inhibited signaling by Wnt-1 CM. Rat-2 cells were either untreated (lane 1) or treated with Wnt-1 (lane 2), Wnt-1 plus Dkk-1-Flag (lanes 3 and 4), Wnt-1 plus mFz8CRD-IgG (lanes 5 and 6), or Wnt-1 plus IgG (lanes 7 and 8). Wnt-1 CM (0.9 ml) were mixed with 1 ml (lanes 3, 5, and 7) or 0.1 ml of the indicated CM balanced with 0.9 ml control CM (lanes 4, 6, and 8). Cytosolic β-catenin was detected via immunoblotting, and relative protein levels are shown. **(b)** Dkk-1-IgG did not deplete Wnt-1 from the Wnt-1/Dkk-1 CM mixture. Wnt-1 (1 ml) was mixed with Dkk-1-IgG (1 ml; lanes 1 and 2) or Dkk-1-AP (1 ml; lanes 3 and 4). The CM mixtures were then depleted with Protein G beads (lanes 1 and 3) or without any manipulation (lanes 2 and 4) and applied to Rat-2 cells (lanes 1–4). For comparison, Rat-2 cells were either untreated (lane 5) or treated with Wnt-1 alone (lane 6). Cytosolic β-catenin was detected via immunoblotting, and relative protein levels are shown (I). CM mixtures after (II, III) or before (IV, V) depletion were immunoblotted with anti-hIgG (II, IV) or Myc (III, V) antibodies (Abs; the AP fusion protein contains a Myc epitope). **(c)** mFz8CRD-IgG, but not Dkk-1-IgG, depleted Wnt-1 activity in Wnt-1 CM. Protein G beads were incubated with mFz8CRD-IgG (lane 1), Dkk-1-IgG (lane 2), IgG (lane 3), or control CM (lane 4). These preloaded beads were incubated with 2 ml of Wnt-1 CM, which after bead removal was applied onto Rat-2 cells. Cytosolic β-catenin was detected via immunoblotting, and relative protein levels are shown. **(d)** Dkk-1 coprecipitated with neither mFz8CRD nor the mFz8CRD-Wnt-1 complex. Wnt-1-Myc (1 ml) was mixed with mFz8CRD-IgG (1 ml; lane 1) or with either mFz8CRD-IgG (1 ml) plus Dkk-1-Flag (1 ml; lane 2) or IgG (1 ml; lane 3). Protein G precipitates (IP; I, III, V) and CM mixtures before precipitation (II, IV, VI) were immunoblotted with anti-Myc (I, II), Flag (III, IV), or hIgG (V, VI) Abs.

Previous experiments have shown a complex formation between the extracellular domains of Fz8 and LRP6 in the presence of Wnt-1 [30]. Strikingly, the Fz8-LRP6 complex induced by Wnt-1 was completely abolished by Dkk-1; that is, mFz8CRD-IgG no longer associated with LRP6N-Myc in a Wnt-1-dependent fashion when either Dkk-1-Flag, Dkk-1-AP, or Dkk-1-IgG but not the control CM was present (Figure 3a and data not shown). The ability of Dkk-1 to disrupt the Fz8-LRP6 complex appeared to be very potent because the disruption occurred even when Dkk-1 in the CM was diluted to a barely detectable level (Figure 3a), likely reflecting the high-affinity interaction observed between Dkk-1 and LRP6. It was shown that LRP6N exhibits Wnt binding activity [30]. Although Dkk-1 did not affect Wnt-1 binding to Fz (Figure 1d), Dkk-1 inhibited Wnt-1 binding to LRP6N (Figure 3b). This finding suggests that Wnt-1 and Dkk-1 binding to LRP6 is mutually exclusive. We also found that LRP5N, the extracellular domain of LRP5 protein that is functionally and structurally related to LRP6, could also associate with mFz8CRD in a Wnt-1-dependent fashion, and this complex formation was abolished by Dkk-1 as well (Figure 3c).

Dkk-1 inhibition of Wnt signaling depends on its ability to bind to LRP6 and to disrupt Fz-LRP6 complex formation

We introduced a single amino acid substitution in Dkk-1 by changing the invariable cysteine residue at amino acid position 220 into alanine (C220A). In transient cotransfec-

Figure 2

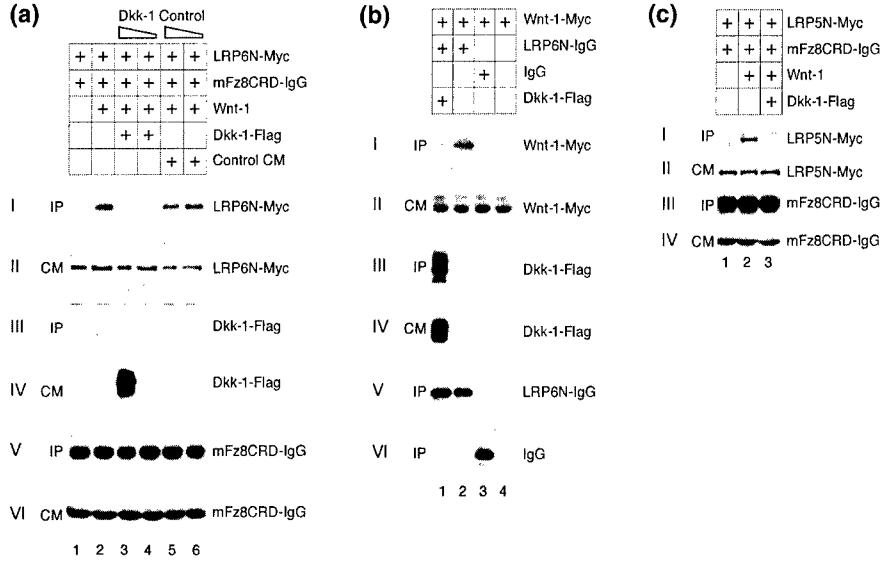


Dkk-1 is a high-affinity ligand for LRP6. **(a)** Dkk-1-Flag coprecipitated with LRP6N-IgG, but not mFz8CRD-IgG or LDLRN-IgG. Dkk-1-Flag (1 ml) was mixed with IgG (0.2 ml; lane 2), mFz8CRD-IgG (0.2 ml; lane 3), LRP6N-IgG (1 ml; lane 4), or LDLRN-IgG (1 ml; lane 5). Protein G precipitates (IP; I, III, V) and CM mixtures before precipitation (II, IV, VI) were immunoblotted with anti-Flag (I, II) or hIgG (III to VI) Abs. Background bands in panels V and VI probably reflect aberrant IgG fusion proteins from pRK5-based vectors. **(b)** LRP6N-Myc, but not LDLRN-Myc, coprecipitated with Dkk-1-IgG. 0.5 ml of Dkk-1-IgG was mixed with either 0.5 ml of control CM (lane 1), 0.5 ml of LRP6N-Myc (lane 2), 0.5 ml of LDLRN-Myc (lane 3), or 0.25 ml each of LRP6N-Myc and LDLRN-Myc (lane 4). Protein G precipitates (IP; I, III) or CM mixtures before precipitation (II, IV) were immunoblotted with anti-Myc (I, II) or hIgG (III, IV) Abs. **(c)** Dkk-1-AP binding to LRP6N-IgG (circles, line) or IgG (squares). The horizontal axis shows Dkk-1-AP concentration (nM); the vertical axis shows changes in absorbency at 405 nm per hour. The insert is the binding data presented as a Scatchard plot.

tion of 293T cells, Dkk-1(C220A) failed to inhibit Wnt-1 induction of β-catenin as Dkk-1 did (Figure 4a), despite their similar secretion into CM. Similarly, Dkk-1(C220A) CM lost the ability to block β-catenin induction by Wnt-1 CM, even when it was present at a 5-fold molar excess relative to the amount of the wild-type Dkk-1 that effectively inhibits Wnt-1 (Figure 4b). Therefore, Dkk-1(C220A) behaved as a loss-of-function mutation in the β-catenin stabilization assay. When tested for binding to LRP6 or LRP5, Dkk-1(C220A) exhibited little, if any, interaction with both proteins in coimmunoprecipitation assays (Figure 4c,d). Most importantly, Dkk-1(C220A) lost the ability to disrupt Wnt-1-induced association between mFz8CRD and LRP6N, even when it was present at 20-fold the concentration at which the wild-type Dkk-1 effectively inhibits the Fz8-LRP6 association (Figure 4e). Therefore, Dkk-1 inhibition of Wnt signaling strictly correlates with its ability to bind to LRP6 and to disrupt Fz-LRP6 complex formation.

We tested whether head induction by Dkk-1 depends on Dkk-1's ability to disrupt the Fz-LRP6 complex. Dkk-1 when combined with inhibition of BMP signaling induces head formation, whereas inhibition of BMP signaling alone induces trunk development [13]. When RNAs for Dkk-1 and a dominant-negative BMP receptor, tBR [34], were coinjected ventrally into the embryo, secondary head induction was seen in more than 78% of the injected embryos, whereas injection of tBR RNA alone only resulted in trunk induction (Figure 5a,b). Dkk-1(C220A) lacked head-inducing capability since coinjection of Dkk-1(C220A) plus tBR RNAs only led to secondary trunk development, as did the injection of tBR RNA alone (Figure 5a,b). We further compared Dkk-1 and Dkk-1(C220A) in the inhibition of Wnt signaling by using the axis duplication assay. Wnt-1 RNA injection mimics activation of the maternal β-catenin pathway and induces ectopic Spemann's organizer formation and axis duplication [1]. While coinjection of RNAs for Wnt-1 and Dkk-1 almost completely suppressed axis induction by Wnt-1, Dkk-1(C220A) was much less effective at inhibiting Wnt-1-induced axis duplication (Figure 5c,d). Some residual effect of Dkk-1(C220A) on Wnt-1 in this sensitive assay suggests that Dkk-1(C220A) may not represent a null mutation. Nonetheless, these results indicate that the ability of Dkk-1 to induce head formation and to antagonize Wnt signaling in embryos correlates with its ability to bind LRP6 and to disrupt Fz-LRP6 complex formation.

Figure 3



Dkk-1 blocks Fz-LRP5/6 complex formation induced by Wnt-1. **(a)** Dkk-1-Flag prevents Wnt-1-induced mFz8CRD-LRP6N complex formation. LRP6N-Myc (0.6 ml) and mFz8CRD-IgG (0.25 ml) were mixed without or with 1 ml of Wnt-1 (lanes 1 and 2), with 1 ml of Wnt-1 plus 2 ml or 0.1 ml of Dkk-1-Flag (lanes 3 and 4), or with corresponding volumes of control CM (lanes 5 and 6). Protein G precipitates (IP; I, III, V) and CM mixtures before precipitation (II, IV, VI) were immunoblotted with anti-Myc (I, II), Flag (III, IV), or hIgG (V, VI) Abs. Note the effectiveness of Dkk-1-Flag CM even when it was deluted to a barely detectable level (lane 4). **(b)** Dkk-1-Flag inhibits Wnt-1-LRP6N interaction. Five milliliters of LRP6N-IgG (lanes 1 and 2), control IgG (lane 3), or control CM (lane 4) was incubated with Protein G beads. Incubation with either 1.5 ml Dkk-1-Flag and 1.5 ml Wnt-1-Myc (lane 1) or 1.5 ml control CM and 1.5 ml Wnt-1-Myc (lanes 2 to 4) followed. Protein G precipitates (IP; I, III, V, VI) and CM mixtures (II, IV) were immunoblotted with anti-Myc (I, II), Flag (III, IV), or hIgG (V, VI) Abs. For the detection of LRP6N-IgG (about 200 Kd) and the control IgG (35 Kd), the same sample was divided into two parts that were separated by 5.5% and 12% gels, respectively. Equal exposures to X-ray films were applied. **(c)** Dkk-1-Flag prevents Wnt-1-induced mFz8CRD-LRP5N complex formation. LRP5N-Myc (1 ml) and mFz8CRD-IgG (0.7 ml) either were mixed without (lane 1) or with (lane 2) 1 ml of Wnt-1 or were mixed with 1 ml of Wnt-1 plus 0.5 ml of Dkk-1-Flag (lane 3). Protein G precipitates (IP; I, III) and CM mixtures before precipitation (II, IV) were immunoblotted with anti-Myc (I, II) or hIgG (III, IV) antibodies.

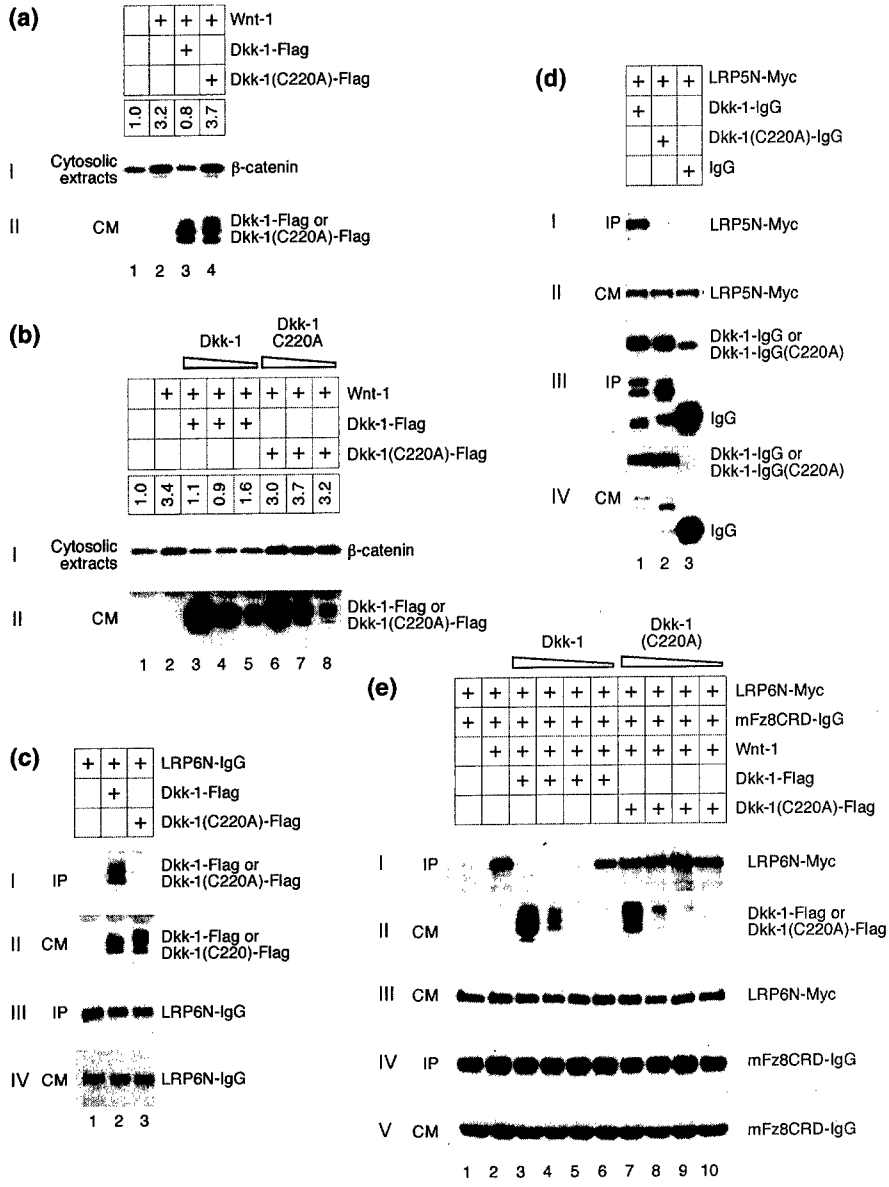
LRP6 and Dkk-1 may be specific for the Wnt/β-catenin pathway

Wnt/Fz signaling also activates β-catenin-independent pathways, such as the conserved planar cell polarity (PCP) pathway that regulates convergent extension movements during vertebrate gastrulation [35–39]. Interestingly, while the arrow/LRP6 mutation causes complete loss of Wnt (Wingless)/β-catenin signaling in *Drosophila*, the Fz PCP pathway appears, by most criteria, to be functional [29]. This raises the possibility that LRP6 is required specifically for Wnt/Fz signaling to the β-catenin pathway. However, this interpretation is uncertain because it is not clear whether Fz PCP signaling in *Drosophila* is activated by any Wnt [40]. Wnt-11 appears to be involved in the Fz PCP pathway in vertebrates [36, 38]. We examined whether LRP6 function is required for Wnt/Fz PCP signaling by using an established explant assay. Animal pole explants differentiate into round-shaped epidermal tissue when cultured alone, but in the presence of mesoderm inducers such as activin, they become dorsal mesoderm, which exhibits morphogenetic elongation movements characteristic of gastrulation movements in vivo [41–43]. This morphogenetic elongation depends on Wnt-11/Fz PCP signaling independent of β-catenin function [35, 37, 38, 44]. A dominant-negative Fz molecule, hFz5N [45], completely inhibited explant elongation (Figure 6a) but not mesoderm induction by activin as assayed by brachyury (Xbra) expression (Figure 6b). This result confirms that this movement requires Wnt/Fz signaling as previously shown [27, 35, 37, 46]. LRP6ΔC minimally affected elongation (Figure 6a), despite the fact that hFz5N and LRP6ΔC were equally effective in the inhibition of Xnr3 induction by Wnt/β-catenin signaling (Figure 6b). These results suggest that LRP6ΔC inhibited the Wnt/Fz/β-catenin pathway but not the Wnt/Fz/PCP pathway, whereas hFz5N inhibited both pathways. Furthermore, Dkk-1 exhibited little inhibition on explant elongation (Figure 6a), while it was a potent inhibitor of Xnr3 induction by Wnt/β-catenin signaling (Figure 6b). Like hFz5N, LRP6ΔC or Dkk-1 exhibited little, if any, inhibition of mesoderm induction by activin (Figure 6b). In addition, Wnt signaling may directly or indirectly activate a calcium-/camodulin-dependent protein kinase II (CaMKII) pathway [47]. Neither LRP6 nor Dkk-1 exhibited any effect on Wnt-5A-induced CaMKII autophosphorylation (data not shown). These results are consistent with the possibility that LRP6 function and Dkk-1 inhibition are specific for the Wnt/β-catenin pathway.

Discussion

In this study, we provided evidence that head inducer Dkk-1 is a high affinity ligand for Wnt coreceptor LRP6 and inhibits Wnt signaling by binding to LRP6 and preventing Fz-LRP6 complex formation. LRP6 function and Dkk-1 inhibition appear to be specific for Wnt/Fz signaling toward the β-catenin pathway. These results have

Figure 4



Dkk-1 activity to inhibit Wnt signaling correlates with its ability to bind LRP5/6 and to disrupt Fz-LRP5/6 complex formation. **(a)** Dkk-1(C220A) failed to inhibit β-catenin stabilization by Wnt-1 in a transient transfection assay. 293T cells were mock transfected (lane 1) or transfected with either 0.5 μg of Wnt-1/LNC alone (lane 2) or Wnt-1/LNC plus 5 μg of either Dkk-1-Flag/CS2+ (lane 3) or Dkk-1(C220A)-Flag/CS2+ (lane 4). Cytosolic β-catenin was detected via immunoblotting, and relative protein levels are shown (I). Dkk-1 or the mutant was detected in the CM of transfected cells (II). **(b)** Dkk-1(C220A) CM failed to inhibit signaling by Wnt-1 CM. Rat-2 cells were untreated (lane 1) or treated with either 1 ml of Wnt-1 alone (lane 2), 1 ml of Wnt-1 plus 1 ml, 0.4 ml, or 0.2 ml of Dkk-1-Flag (lanes 3 to 5), or 1 ml of Wnt-1 plus 1 ml, 0.4 ml, or 0.2 ml of Dkk-1(C220A)-Flag (lanes 6 and 7). Cytosolic β-catenin was detected via immunoblotting, and relative protein levels are shown (I). CM mixtures were immunoblotted with the anti-Flag Ab (II). **(c)** Dkk-1-Flag, but not Dkk-1(C220A)-Flag, was coprecipitated with LRP6N-IgG. LDP6N-IgG CM (3 ml) was mixed with either 1 ml of control CM (lane 1), 1 ml of Dkk-1-Flag (lane 2), or 1.5 ml of Dkk-1(C220A)-Flag (lane 3). Protein G precipitates (IP; I, III) and CM mixtures before precipitation (II, IV) were immunoblotted with anti-Flag (I, II) or hIgG (III, IV) Abs. **(d)** LRP5N-Myc was coprecipitated with Dkk-1-IgG but not with Dkk-1(C220A)-IgG. LRP5N-Myc (1 ml) was mixed with 2 ml of either Dkk-1-IgG (lane 1), Dkk-1(C220A)-IgG (lane 2), or control IgG (lane 3). Protein G precipitates (IP; I, III) and CM mixtures before precipitation (II, IV) were immunoblotted with anti-Myc (I, II) or hIgG (III, IV) Abs. **(e)** Dkk-1(C220A)-Flag failed to disrupt mFz8CRD-LRP6N complex formation induced by Wnt-1. LRP6N-Myc (0.8 ml) and mFz8CRD-IgG (0.5 ml) were mixed without (lane 1) or with 1.3 ml of Wnt-1 (lanes 2–10) in the presence of 1 ml, 0.2 ml, 0.05 ml, or 0.01 ml of Dkk-1-Flag (lanes 3–6) or Dkk-1(C220A)-Flag (lanes 7–10). Protein G precipitates (IP; I, IV) and CM mixtures before precipitation (II, III, V) were immunoblotted with anti-Myc (I, III), Flag (II), or hIgG (IV, V) Abs.

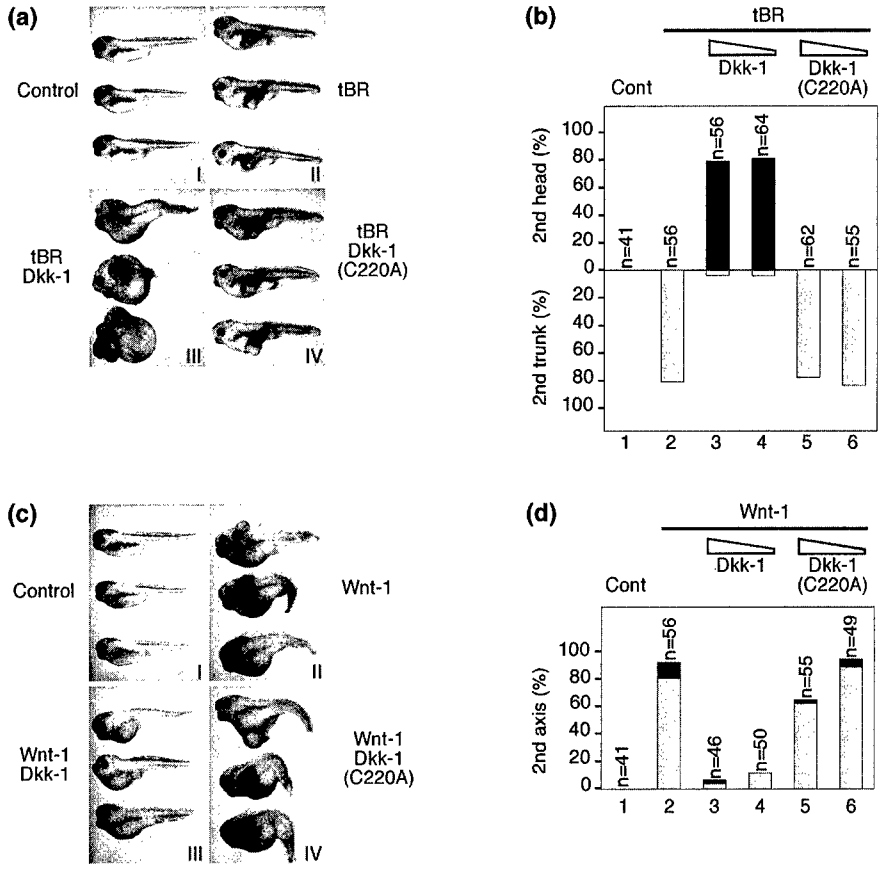
important implications for our understanding of Dkk-1 function in head induction, of the Fz-LRP6 complex in Wnt signal transduction, and of mechanisms of Wnt signaling modulation and specificity.

Dkk-1 antagonizes Wnt signaling by blocking Fz-LRP6 complex formation

Previously characterized Wnt antagonists, such as Cerberus, WIF-1, and members of the Frzb/sFRP family, are Wnt binding proteins that presumably function by competing with Fz for Wnt ligands [11, 12, 14, 18, 26, 27]. In fact, Frzb proteins share the same structural motif, the cysteine-rich domain (CRD), that functions as the Wnt binding domain in Fz receptors [11, 12, 25]. Although Dkk-1 functions as a potent inhibitor of Wnt signaling, Dkk-1 does not possess significant Wnt binding activity, as demonstrated by its inability to deplete Wnt from conditioned medium. Thus, Dkk-1 likely achieves its inhibition by blocking Wnt receptor function.

Although it is established that Fz proteins are Wnt receptors [24], LRP6, a single-pass transmembrane receptor be-

Figure 5



The ability of Dkk-1 to induce head formation correlates with its ability to disrupt Fz-LRP6 complex formation. Embryos were scored at stage 36. Representative embryos are shown in **(a)** and **(c)**; an overall tabulation of results is depicted in **(b)** and **(d)**. (a,b) Dkk-1, but not Dkk-1(C220A), induced head formation when coexpressed with a dominant-negative BMP receptor (tBR). Embryos were injected ventro-vegetally with 20 pg tBR RNA alone or 20 pg tBR RNA plus either 5 or 2.5 pg of RNA for Dkk-1 or Dkk-1(C220A). (c,d) Dkk-1(C220A) did not effectively inhibit dorsal axis duplication by Wnt-1. Embryos were injected ventro-vegetally with 10 pg of Wnt-1 RNA alone or 10 pg of Wnt-1 RNA plus 5 or 2.5 pg of Dkk-1 or Dkk-1(C220A) RNA. The more darkly shaded area represents axis duplication with the cement gland and at least one eye. The lighter shading represents partial axis duplication.

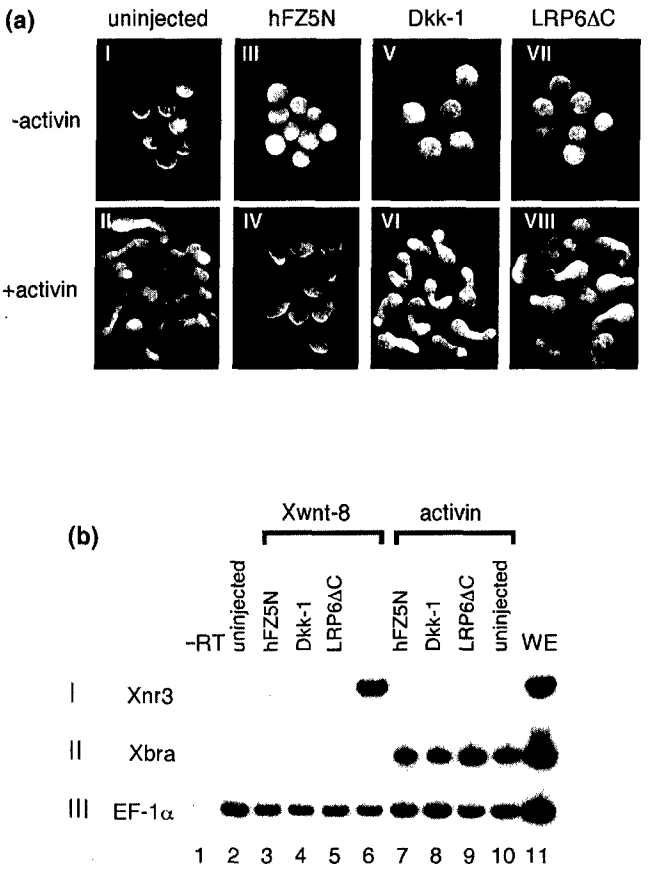
longing to the LDL receptor family, is also critical for Wnt signal transduction. Genetic studies in *Drosophila* indicate that the LRP6 homolog encoded by the *arrow* gene is essential for Wingless signaling [29]. Similarly, mice lacking the *lrp6* gene exhibit developmental phenotypes associated with mutations of multiple Wnt genes [31]. In the *Xenopus* embryo, inhibition of LRP6 function blocks Wnt signaling, whereas overexpression of LRP6 mimics it [30]. The observation that the LRP6 extracellular domain can bind Wnt-1 and associate with Fz8CRD in the presence of Wnt-1 [30] suggests that LRP6 functions as a Wnt coreceptor for Fz proteins. Our current study on how Dkk-1 antagonizes Wnt signaling provides significant support for this hypothesis. We show that Dkk-1 is a high-affinity ligand for LRP6 and prevents Fz-LRP6 complex formation that is induced by Wnt-1. In addition, Dkk-1 also binds to LRP5, which is the only identifiable close relative of LRP6 in the sequenced human genome, and disrupts Wnt-1-induced Fz-LRP5 complex formation. Notably, Dkk-1 does not exhibit any effect on the Wnt-Fz interaction, and Dkk-1 inhibition of Wnt signaling strictly correlates with its ability to bind LRP5/6 and to disrupt Fz-LRP5/6 association induced by Wnt. Therefore, Fz-LRP5/6 complex formation is most likely the target of Dkk-1 action. Furthermore, these results also suggest that it is Wnt-Fz-LRP5/6 complex formation, but not Wnt-Fz interaction, that triggers Wnt/β-catenin signaling, and they provide a molecular explanation for LRP6 requirement in Wnt signal transduction.

The binding affinity between Dkk-1 and LRP6 is about 0.5 nM and thus is significantly higher than Wnt-Fz binding affinities of 5–9 nM [32, 48]. Consistent with the high-affinity interaction between Dkk-1 and LRP6, Dkk-1 inhibits Wnt-1-LRP6 interaction, but not Wnt-1-Fz interaction, and efficiently blocks Wnt-dependent Fz-LRP6 complex formation. It remains to be investigated whether Dkk-1 and Wnt compete for the same binding site on LRP6 or whether Dkk-1 binding to LRP6 results in conformational changes that prevent LRP6 interaction with Wnt and with the Wnt-Fz complex.

Head induction, gastrulation, and Wnt signaling specificity: why does Spemann's organizer secrete so many Wnt antagonists?

In *Xenopus* embryos, head induction can be achieved by simultaneous inhibition of Wnt and BMP signaling, whereas trunk induction requires inhibition of BMP and activation of Wnt signaling [9, 13]. Indeed, many inducing molecules secreted by Spemann's organizer are either Wnt antagonists, which include Dkk-1, Frzb (sFRP-3), sFRP-2, and Crescent [11–17], or BMP antagonists, such as Chordin,

Figure 6



LRP6 function and Dkk-1 inhibition may be specific for the Wnt/β-catenin pathway. **(a)** activin-induced elongation movements of animal pole explants were significantly inhibited by hFZ5N, but not by LRP6ΔC or Dkk-1. Two-cell-stage embryos were injected into the animal region with 2 ng of hFZ5N or LRP6ΔC RNA or with 500 pg of Dkk-1 RNA. Animal pole explants were dissected at stage 9 and treated without (I, III, V, VII) or with (II, IV, VI, VIII) 5 ng/ml activin. **(b)** hFZ5N, LRP6ΔC, and Dkk-1 were effective in blocking Xnr3 induction by Xwnt-8/β-catenin signaling and did not inhibit mesoderm induction by activin. hFZ5N, Dkk-1, or LRP6ΔC (as in [a]) abolished Xnr3 induction by Xwnt-8 RNA (10 pg; I, lanes 3–6) but did not inhibit Xbra induction by activin (II, lanes 7–10). EF-1α was used as loading control (III). "WE" stands for "whole embryo," and "-RT" stands for "without reverse transcriptase."

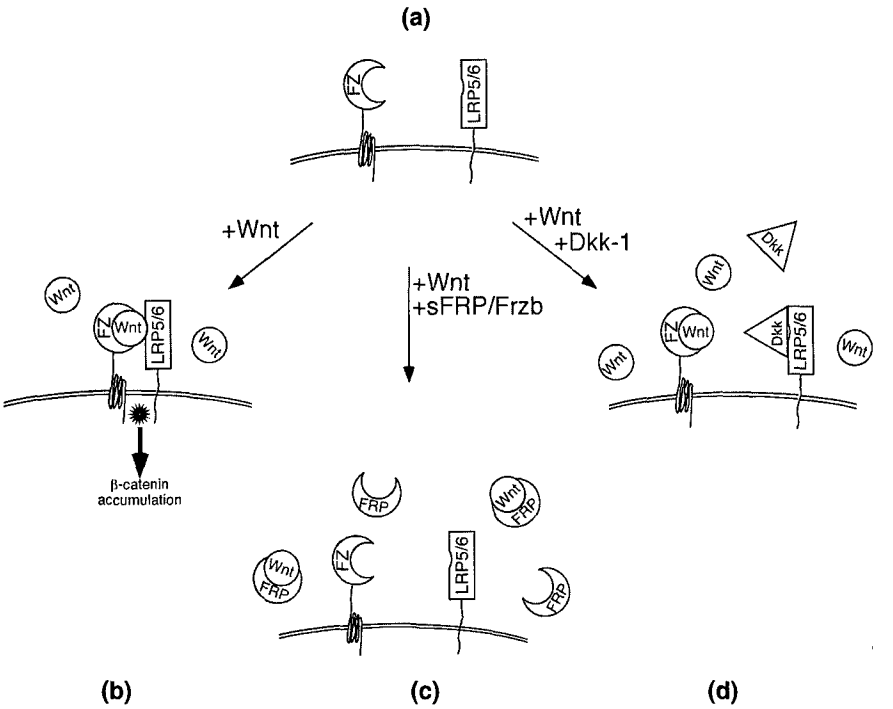
Noggin, and Follistatin [49–51]. In addition, the head inducer Cerberus inhibits signaling by Wnt, BMP, and Nodal [10, 14]. The Wnt antagonists are expressed in regions known to be associated with head-inducing activities and function as head/anterior inducers when tested in the embryo [9, 13, 18]. Furthermore, depletion of Dkk-1 function in *Xenopus* embryos by a blocking antibody results in headless or cyclopic embryos [13, 52]. This finding demonstrates an essential role of Dkk-1 in head formation and further suggests nonredundant functions for at least some of these organizer-specific Wnt antagonists.

Wnt signal transduction regulates several critical steps in early vertebrate embryogenesis via distinct signaling pathways. Wnt/Fz signaling via β-catenin governs Spemann's organizer formation [1, 5, 6] and head/trunk induction (see below). Wnt/Fz signaling also regulates morphogenetic movements during gastrulation and neurulation via a β-catenin-independent pathway that is similar to the Fz planar cell polarity (PCP) pathway in *Drosophila* [40, 53, 54]. It is unknown how Wnt/Fz signaling specificity toward different pathways is governed. We demonstrate that while dominant-negative Fz and LRP6 mutants, hFz5N and LRP6ΔC, respectively, are equally effective in inhibiting Wnt/β-catenin signaling, only hFz5N, but not LRP6ΔC, fully blocks gastrulation movements that are controlled by the Wnt/Fz PCP pathway. Therefore, LRP6 function appears to be specifically required for the Wnt/Fz/β-catenin pathway. Further supporting this idea is the fact that Dkk-1, which binds LRP6, exhibits little effect on Wnt/Fz PCP signaling and gastrulation movements but inhibits gene induction by β-catenin signaling. Therefore, Dkk-1 appears to be a specific antagonist for the Wnt/β-catenin pathway.

An interesting comparison may be made between Dkk-1 and Wnt binding antagonists. Frzb and Crescent inhibit Wnt/β-catenin signaling in axis induction and, with different efficacy, Wnt/PCP signaling during gastrulation [15, 17]. The distinct mechanisms and inhibition spectra of these Wnt antagonists may, in part, explain why Dkk-1, Frzb, and other organizer-derived antagonists have overlapping but not redundant functions [13, 52]. When coinjected with tBR, Dkk-1 induces complete head development with bilateral eyes, whereas Frzb induces the development of a head with a cyclopic eye [13, 18]. We speculate that if Frzb inhibits both Wnt/β-catenin and Wnt/PCP pathways, Frzb may interfere with anterior extension of the prechordal plate, which is required for the separation of the single eye field into two eyes [55, 56]. Our findings further suggest that it is the Wnt/β-catenin pathway that Dkk-1 antagonizes to allow head development. This is in full agreement with genetic analyses of the zebrafish *headless* gene, which when mutated generates anterior-deficient embryos and encodes a TCF (T cell factor) transcription repressor that inhibits β-catenin-dependent gene expression [57].

Dkk-1 is the founding member of the Dkk family, which includes Dkk-2, Dkk-3, and Dkk-4 [13, 20]. Dkk genes, like Wnt genes or genes encoding other Wnt antagonists, are expressed in highly dynamic patterns during vertebrate development and in specific adult tissues [21]. In the axis duplication assay, Dkk-1 and Dkk-4 antagonize Wnt signaling, whereas Dkk-2 and Dkk-3 do not [20]. In fact, one study suggests that Dkk-2 can antagonize Dkk-1 and thus may play a positive role in Wnt signaling [58]. Further studies are needed to provide an understanding of how Dkk proteins, as well as other LRP5/6 ligands such

Figure 7



A model for the Fz-LRP5/6 complex that is induced by Wnt and differentially modulated by sFRP/Frzb and Dkk-1. **(a)** Fz and LRP5/6 do not associate in the absence of Wnt. **(b)** Wnt induces the formation of the Fz-LRP5/6 complex that triggers Wnt/β-catenin signaling. **(c)** sFRP/Frzb proteins, Cerberus, WIF-1 and other Wnt binding antagonists bind specific Wnt molecules and prevent Wnt interaction with Fz and/or LRP5/6; they thereby prevent formation of the Fz-LRP5/6 complex. (sFRP/Frzb may also bind certain Fz proteins, not depicted here). These antagonists may inhibit multiple Wnt/Fz signaling pathways, depending on the specific Wnt or Fz proteins to which they bind. **(d)** Dkk-1 binds to LRP5/6 and prevents Fz-LRP5/6 complex formation but does not affect the Wnt-Fz interaction. Thus, Dkk-1 may specifically inhibit LRP5/6-dependent signaling, such as the β-catenin pathway. We emphasize that this working hypothesis remains to be fully tested.

as ApoE, interact with LRP5/6 to regulate Wnt signaling. Nonetheless, Dkk modulation of Wnt signaling in a LRP5/6-specific and probably pathway-specific manner complements the action of Wnt binding antagonists that regulate Wnt signaling in a Wnt- or Fz-specific manner (Figure 7). Together, these Wnt antagonists provide a comprehensive network that permits precise and fine modulation of Wnt signal transduction pathways during vertebrate embryogenesis.

Materials and methods

cDNA construction

Human Dkk-1 cDNA was used in this study. Plasmids Dkk-1-Flag/CS2+ [20], mFz8CRD-IgG/pRK5, IgG/pRK5 [32], LRP6N-IgG/pcDNA3.1+, LRP6N-Myc/pcDNA3, LRP6/CS2+, LRP6ΔC/CS2+ [30], LDLR-FC/pcDNA3.1+ [59], Wnt-5A-Myc/pSP64T [60], tBMPR/pSP64T [61], and hFZ5N/CS2+ [45] have been described. LDLRN-Myc/pcDNA3 and LRP5N-Myc/pcDNA3 were generated by fusion of the extracellular domains of LDLR or LRP5 with the 6-Myc epitope (from CS2+MT). Dkk-1-IgG/pcDNA3.1+ and Dkk-1-AP/APtag5 constructs contain the full-length Dkk-1 fused with the IgG tag from IgG/pRK5 [32] or alkaline phosphatase [62]. The C220A mutation was introduced into Dkk-1 cDNA by primer-mediated PCR mutagenesis and verified via DNA sequencing. Details of these plasmids are available upon request.

Cell culture, CM production, and cytosolic β-catenin protein assay

Rat-2 and 293T cell lines were maintained in high-glucose DMEM medium with 10% fetal bovine serum. All recombinant proteins were used as conditioned media (CM) and, with the exception of Wnt-1 and Wnt-1-Myc, were produced in 293T cells via transient transfection with the indicated expression vectors by the calcium phosphate method. Wnt-1 and Wnt-1-Myc CM were collected from retrovirally infected Rat-2 cells and analyzed for activity in a cytosolic β-catenin induction assay. Collected CM was cleared of cellular debris by centrifugation, aliquoted, and frozen at −80°C.

Rat-2 cells on 6-well tissue culture plates were treated with various CM mixtures for 2.5-3 hr. Cytosolic extracts were immunoblotted for β-catenin as described [63]. The equal amounts of protein loading (usually 10–20 μg per line) were confirmed by the silver staining of test gels. β-catenin protein levels were measured with IPLab Gel image processing software on 12 bit scans on immunoblotting films. The β-catenin level in untreated cells was set as 1.0. Numbers presented were averages from 2 to 4 independent experiments.

Antibodies and immunoblotting

Protein samples were resolved by SDS-polyacrylamide gel electrophoresis and transferred onto Immobilon-P membrane (Millipore). For the detection of LRP6N, LRP5N, and LDLRN recombinant proteins, 5.5% gels were used, and 8% and 12% gels were used for the detection of β-catenin and all other proteins, respectively. Membranes were blocked with TBST (10 mM Tris [pH 7.4], 150 mM NaCl, 0.05% Tween 20) containing 5% nonfat dry milk for 2 hr at room temperature or overnight at +4°C. The following antibodies were used for immunoblotting according to manufacturers' guidelines: Flag (M2, Sigma), β-catenin (Transduction Labotatories), Myc (9E10, Santa Cruz Biotechnology), hIgG-Fc-specific HRP conjugate, and mouse IgG HRP conjugate (Jackson Laboratories). Renaissance chemiluminescence reagents (NEN) were used for immunoblot detection.

Protein G precipitation and depletion

Combinations of CM were mixed and equalized in total volume by the use of control medium, and 10 μl of Protein G agarose beads (Pharmacia) was added to the mixture. After 2 hr of incubation on a nutator at room temperature, precipitates on beads were washed four times with PBS or TBS with 0.05% Tween 20 and then boiled for 3 min in 50 μl of loading buffer before separation by gel electrophoresis.

For the depletion of Wnt-1 from Wnt-1 CM, 50 μl of Protein G beads were incubated and preloaded with 20 ml of CM containing either Dkk-1-IgG, mFz8CRD-IgG, or control IgG. The beads were washed two times with PBS and were added to 2 ml of Wnt-1 CM for incubation for 30 min. After bead removal by centrifugation, the remaining CM was added to Rat-2 cells.

Affinity measurement

The amount of LRP6N-IgG, control IgG, and Dkk-1-AP (which contains a single Myc epitope) in the CM was determined by immonoblotting, with pure human IgG (Sigma) and a recombinant protein with a single Myc epitope (provided by R. Habas) as standards, respectively. CM mixtures (1 ml) containing 0.25 nM LRP6N-IgG or 5 nM control IgG plus various concentrations (0–22 nM) of Dkk-1-AP were incubated with 10 μl of Protein G agarose beads for 4 hr at room temperature. The Protein G beads were washed four times with PBS with 0.05% Tween 20. After a complete decanting, 1 ml of alkaline buffer with p-nitrophenyl phosphate was added, and a change in absorbency at 405 nm was measured. To calculate the bound/free ratio for Dkk-1-AP, the activity of bound Dkk-1-AP on beads was divided by the activity of Dkk-1-AP in the CM before binding.

Embryological methods

Xenopus eggs were fertilized in vitro and cultured in 0.1× Marc's modified ringer's medium (MMR). Capped synthetic mRNAs for embryo injection were synthesized in vitro with the mMessage mMachine kit (Ambion). For secondary axis and head induction assays, embryos were injected with synthetic RNAs ventrally at the four-to-eight-cell stage. Results from at least two independent experiments were combined.

For the explant elongation assay, two-cell-stage embryos were injected with synthetic RNAs at the animal pole region. Animal poles were dissected at stage 9 and cultured in 0.5× MMR containing 1% BSA with or without 5 ng/ml of human activin A [kindly provided by Dr. Eto (Ajinomoto Co.)]. Elongation was scored at stage 17. Expression of Xnr3, Xbra, and EF-1α was examined at stage 10.5 by RT-PCR as previously described [64].

Note added in proof

While this manuscript was being revised, it was reported that the LRP5 cytoplasmic domain binds Axin (Mao J, Wang J, Liu B, Pan W, Farr GH, Flynn C, *et al*: Low-density lipoprotein receptor-related protein 5 binds to Axin and regulates the canonical Wnt signaling pathway. *Mol Cell* 2001, 7:801-809).

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Rescue of a telomere length defect of Nijmegen breakage syndrome cells requires NBS and telomerase catalytic subunit

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Nijmegen breakage syndrome (NBS) is a rare human disease displaying chromosome instability, radiosensitivity, cancer predisposition, immunodeficiency, and other defects [1, 2]. NBS is complexed with MRE11 and RAD50 in a DNA repair complex [3–5] and is localized to telomere ends in association with TRF proteins [6, 7]. We show that blood cells from NBS patients have shortened telomere DNA ends. Likewise, cultured NBS fibroblasts that exhibit a premature growth cessation were observed with correspondingly shortened telomeres. Introduction of the catalytic subunit of telomerase, TERT, was alone sufficient to increase the proliferative capacity of NBS fibroblasts. However, NBS, but not TERT, restores the capacity of NBS cells to survive γ irradiation damage. Strikingly, NBS promotes telomere elongation in conjunction with TERT in NBS fibroblasts. These results suggest that NBS is a required accessory protein for telomere extension. Since NBS patients have shortened telomeres, these defects may contribute to the chromosome instability and disease associated with NBS patients.

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Results and discussion

We investigated whether the vulnerability of Nijmegen breakage syndrome (NBS) cells to chromosome instability could be associated with altered telomere dynamics. Blood mononuclear cell DNAs were isolated from affected patients and their family members for the typical 657del5 NBS frameshift mutation [8] and from unaffected families. These samples were analyzed by restriction enzyme digestion, fractionation by gel electrophoresis, and *in gel* hybridization to telomere repeats using a telomere-specific probe as previously described [9]. We examined samples from NBS patients with an age ranging from 7 to 18 years and unaffected individuals ranging from 9 to 46 years of age. Quantitation of the Terminal Restriction Fragment (TRF) differences indicated that the telomeric DNA for NBS patients was significantly shorter. NBS patients yielded a mean TRF of 7.77 kb compared with 10.32 kb for normal patients ($p = 0.0032$; Figure 1a). NBS heterozygotes were not significantly different from the TRF values for the unaffected patients; although, the data was insufficient to monitor TRF as a function of age (data not shown). These results are suggestive of a strong correspondence between reduced telomere length and the diverse clinical symptoms in NBS patients, including a profound rate of malignancies at an early age [10, 11]. Previous indications of errors in telomere metabolism have been observed with ataxia telangiectasia and Werner syndrome patients, also having chromosome anomalies and growth deficiencies [12, 13].

Evidence of shortened telomeres may be attributable to undue expansion of NBS peripheral lymphoid cells prompted by frequent infections or may be formed by an intrinsic defect in telomere regulation. To study potential dynamics of telomere length modulation per se, we investigated primary NBS fibroblasts obtained relatively early in passage history, (GM07166-Passage 8; 880823H-Passage 5 and 780816J-Passage 12). GM07166 and 780816J fibroblasts grew poorly and reached senescence after ~10 population doublings (PD) (Figure 1b). In keeping with the limited proliferative potential, significant telomere decay was already evident for GM07166 and 780816J cells relatively early in their passage history (Figure 1c). 880823H cells had longer telomeres and a correspondingly increased proliferative capacity. Yet, at >25 PD (population doubling), 880823H cells yielded shorter TRF and

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Wnt/Frizzled Activation of Rho Regulates Vertebrate Gastrulation and Requires a Novel Formin Homology Protein Daam1

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Summary

Wnt signaling via the Frizzled (Fz) receptor controls cell polarity and movement during development, but the molecular nature of Wnt/Fz polarity signal transduction remains poorly defined. Here we report that in human cells and during Xenopus embryogenesis, Wnt/Fz signaling activates the small GTPase Rho, a key regulator of cytoskeleton architecture. Wnt/Fz activation of Rho requires the cytoplasmic protein Dishevelled (Dvl) and a novel Formin homology protein Daam1. Daam1 binds to both Dvl and Rho, and mediates Wnt-induced Dvl-Rho complex formation. Inhibition or depletion of Daam1 prevents Wnt/Fz activation of Rho and of Xenopus gastrulation, but not of β-catenin signaling. Our study illustrates a molecular pathway from Wnt/Fz signaling to Rho activation in cell polarity signal transduction.

Introduction

The establishment of cell polarity is a central issue in biology. Cell polarity controls cell division, morphology, movement, and function. While the epithelial apical-basal polarity is well characterized, less is known about the polarity within the epithelia plane, referred to as *p*lanar *c*ell *p*olarity (PCP) (Adler, 1992; Gubb, 1993). An example of PCP is illustrated by the sensory hair cell, which detects sound/motion via graded stereocilia elaborated like a staircase on its apical surface. Disruption of this PCP results in human deafness (Eaton, 1997). Genetic studies of PCP in Drosophila wing and eye development have revealed a group of "core" PCP genes (Shulman et al., 1998; Mlodzik, 1999), most notably *frizzled* (*Dfz1*) and *dishevelled* (*dsh*). *Dfz1* encodes a serpentine receptor that is hypothesized to be a receptor for an unknown extracellular PCP signal, whereas *dsh* encodes a cytoplasmic scaffolding protein (Boutros and Mlodzik, 1999). In vertebrates, Fz/Dsh PCP signaling is essential for cell polarity and movement during gastrulation (Sokol, 2000).

Fz and Dsh families of proteins are components of the conserved Wnt/β-catenin signaling pathway that controls cell fate and proliferation (Wodarz and Nusse, 1998; Miller et al., 1999). Secreted Wnt ligands, upon binding to Fz receptors and the coreceptor LRP5/6 (Wehrli et al., 2000; Tamai et al., 2000; Pinson et al., 2000), signal via Dsh to stabilize β-catenin, leading to the activation of β-catenin-dependent transcription. However, Fz/Dsh PCP function in Drosophila and vertebrates requires neither β-catenin nor other components involved in β-catenin signaling (Strutt et al., 1997; Axelrod et al., 1998; Boutros et al., 1998; Tada and Smith, 2000; Heisenberg et al., 2000; Wallingford et al., 2000), suggesting a distinct transduction pathway. More than a dozen core PCP genes in Drosophila have been identified, many of which encode proteins of unknown functions, including several multi-pass transmembrane proteins (Shulman et al., 1998), implying that PCP regulation is complex. Interestingly, core PCP gene products also include Rho, Rac (Strutt et al., 1997; Boutros et al., 1998; Eaton et al., 1996; Fanto et al., 2000), and the Rho-associated kinase (Drok) (Winter et al., 2001). The Rho family of GTPases, including Rho, Rac, and Cdc42, are molecular switches that regulate cytoskeleton and transcription via cycling between inactive GDP-bound and active GTP-bound forms (Hall, 1998; Kaibuchi et al., 1999). Although *RhoA* genetically interacts with *Dfz1* and *dsh* (Strutt et al., 1997), whether/how Wnt signaling is coupled to Rho function is unknown, and the molecular nature of PCP signal transduction remains obscure.

Here we report that Wnt/Fz signaling directly activates RhoA. Wnt/Fz activation of RhoA requires Dsh and Daam1 (*D*ishevelled-*a*ssociated *a*ctivator of *m*orphogenesis), a novel Formin-homology (FH) protein. Daam1 binds to both Dsh and RhoA, and mediates Wnt-induced Dsh-RhoA complex formation. Daam1 function is essential for Wnt/Fz PCP signaling during Xenopus gastrulation.

Results

Wnt/Fz Signaling Activates Rho

RhoA genetically interacts with *Dfz1*/*dsh* in Drosophila (Strutt et al., 1997), but whether DFz1/Dsh signaling regulates RhoA activity is not known. Previous studies have demonstrated that Rho activation in vivo can be detected via a GST-RBD [*R*ho *b*inding *d*omain, fused with the glutathione S-transferase (GST)] fusion protein that recognizes the GTP-bound Rho (Ren et al., 1999). Using this assay, we found that Fz proteins, upon expression in human 293T cells, exhibit distinct RhoA activation capabilities (Figure 1A). Fz7 is implicated in both the PCP and β-catenin pathways (Sumanas et al., 2000; Medina et al., 2000; Djiane et al., 2000), and indeed activates RhoA (Figure 1A). Fz1 similarly activates RhoA (Figure 1A) and β-catenin signaling (Yang-Snyder et al., 1996) By contrast, Fz5 activates β-catenin signaling (He et al., 1997) but not RhoA, whereas Fz2 activates neither β-catenin nor RhoA (Figure 1A), but may activate a Ca^{2+}-dependent pathway (Sheldahl et al., 1999). Interestingly, Fz1 and Fz7, but not Fz2, can result in Dsh plasma membrane translocation (Rothbacher et al., 2000), which

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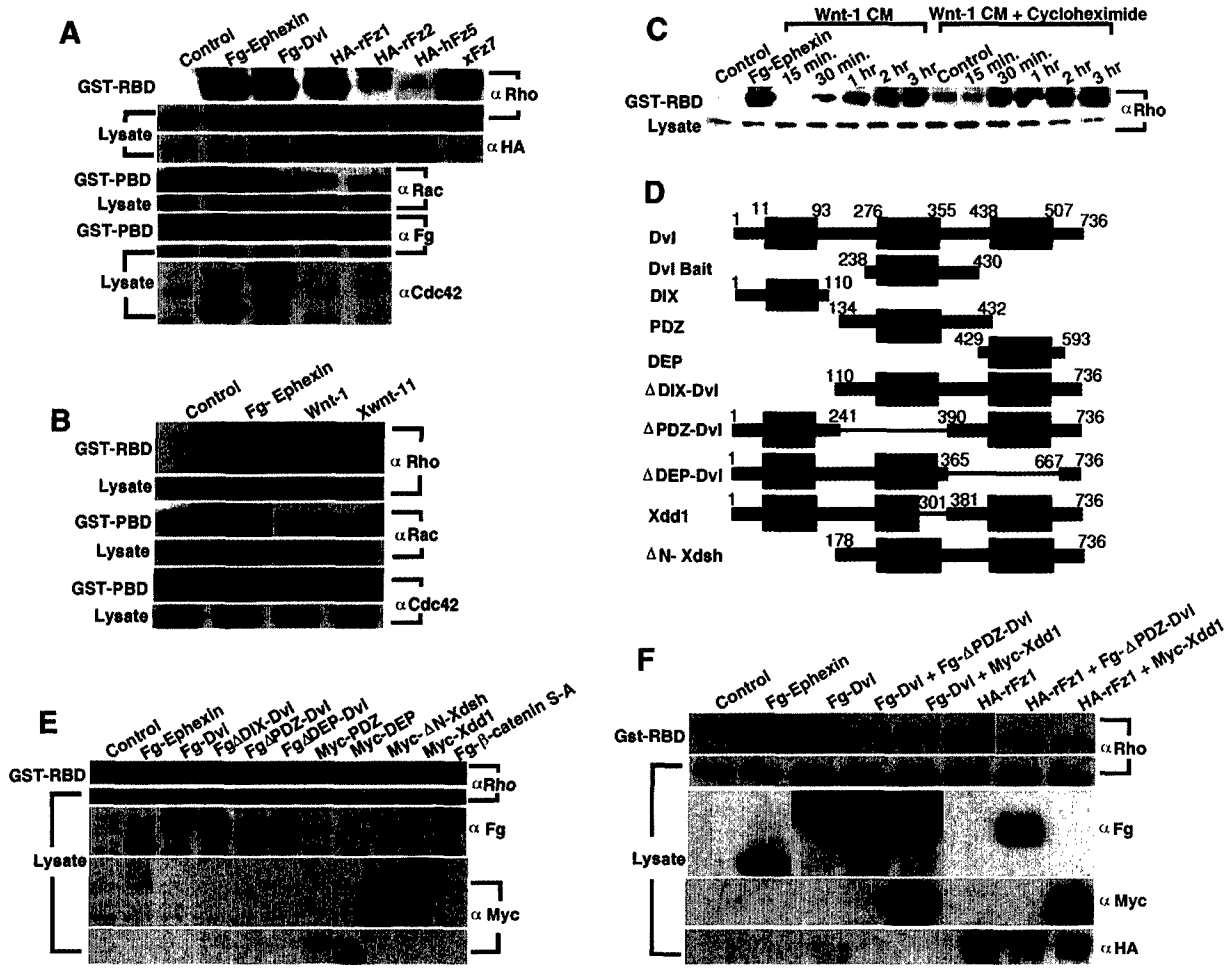

Figure 1. RhoA Is a Direct Downstream Component of Wnt/Fz/Dvl Signaling

(A) Specific Fz proteins and Dvl2 (labeled as Dvl) activate RhoA and possibly Rac, but not Cdc42. Ephexin activates all three GTPases. GTP-bound Rho in cell lysates was precipitated using GST-RBD and detected by RhoA-specific antibodies (Abs). GTP-bound Rac and Cdc42 were precipitated using GST-PBD and detected by Rac- or Cdc42-specific Abs, respectively. Rho, Rac, and Cdc42 in lysates were detected by the above Abs. In all transfection experiments, products from transfected cDNAs were monitored by immunoblotting the lysate with Abs against Flag (Fg), hemagglutinin (HA), or Myc epitope. Fz1 and Fz2 (rat), Fz5 (human), and Fz7 (Xenopus) were used.

(B) Wnt-1 transfection activates RhoA and Rac but not Cdc42. Xenopus Wnt-11 activates none.

(C) Wnt-1 CM, but not control CM, activates RhoA in the absence of new protein synthesis. Cycloheximide does not affect Rho activation, but inhibits cyclinD1 induction (not shown).

(D) Dvl2 and Xdsh constructs. Numbers indicate amino acid positions and the thin line represents internal deletions.

(E) The Dvl2 PDZ and DEP domains, but not the DIX domain, are required for Rho activation. A stabilized β-catenin (S-A) has no effect on Rho activity but causes an 18-fold activation of a TCF-reporter gene (not shown).

(F) Either ΔPDZ-Dvl or Xdd1 blocks Rho activation by Fz or Dvl2.

correlates with Fz/PCP but not Wnt/β-catenin signaling (Axelrod et al., 1998, 2001; but see Boutros et al., 2000). Fz1 also activates Rac weakly but not Cdc42 (Figure 1A), as detected via an analogous assay using GST-PBD (*p21 b*inding *d*omain) that recognizes GTP-bound Rac or Cdc42 (Benard et al., 1999; Akasaki et al., 1999). Ephexin, which is a guanine nucleotide exchange factor (GEF) for Rho, Rac, and Cdc42 (Shamah et al., 2001), activates all three GTPases. Thus, specific Fz signaling activates RhoA and possibly Rac.

Wnt-11 regulates PCP in vertebrates (Tada and Smith, 2000; Heisenberg et al., 2000). We thus examined whether Wnt signaling activates RhoA. Surprisingly, Wnt-1, which has been studied primarily in β-catenin signaling, activates RhoA and Rac, but not Cdc42 (Figure 1B). Importantly, Wnt-1 conditioned medium (CM) induces RhoA activation, which is not blocked by cycloheximide, a protein synthesis inhibitor (Figure 1C). Thus, RhoA lies directly downstream of Wnt signaling. Xenopus Wnt-11 activates none of the GTPases in 293T cells (Figure 1B), possibly due to a lack of appropriate receptors since Wnt-11 activates RhoA in embryos (see below).

Fz/Dsh Activation of Rho Reflects PCP Signaling

Dsh is downstream of Fz signaling, and thus may be involved in RhoA activation. Indeed, mouse Dishevelled-2 (Dvl2) activates RhoA and Rac but not Cdc42 (Figure 1A). Other Dsh proteins, including Dvl1 and Dvl3,

and Xenopus and Drosophila Dsh also activate RhoA (not shown). If RhoA activation by Wnt/Fz and Dvl underlies PCP signaling, Fz activation of RhoA should require Dvl but not β-catenin, and Dvl activation of RhoA should correlate with PCP function. Dsh/Dvl proteins have three conserved domains: the amino DIX domain, the central PDZ domain, and the carboxyl DEP domain (Boutros and Mlodzik, 1999; Figure 1D). The DIX domain is essential for β-catenin signaling but dispensable for PCP, whereas both the PDZ and DEP domains are required for PCP function (Axelrod et al., 1998; Boutros et al., 1998; Tada and Smith, 2000; Heisenberg et al., 2000; Wallingford et al., 2000). We tested a panel of Dvl2 mutants (Figure 1D) for RhoA activation. ΔDIX-Dvl (lacking the DIX domain) is fully capable of activating RhoA, but neither ΔPDZ-Dvl (lacking the PDZ domain) nor ΔDEP-Dvl (lacking the DEP domain) is able to do so (Figure 1E). We also tested two Xenopus dsh mutants. ΔN-Xdsh (lacking the DIX domain) is functional in PCP signaling, and indeed activates RhoA, whereas Xdd1 (lacking a part of the PDZ domain) activates neither PCP signaling (Sokol, 1996; Tada and Smith, 2000; Heisenberg et al., 2000; Wallingford et al., 2000) nor RhoA (Figure 1E). Further, Xdd1 is a dominant negative inhibitor for the PCP pathway (Sokol, 1996), and indeed inhibits RhoA activation by Dvl2 (Figure 1F). Thus, we observed a full correlation between Dvl activation of RhoA and its cell polarity function.

We also examined whether Dsh mediates Fz activation of RhoA. We found that Xdd1 or ΔPDZ-Dvl blocks Fz activation of RhoA (Figure 1F), whereas neither β-catenin nor GSK-3 inhibition, which stabilizes β-catenin, activates RhoA or interferes with RhoA activation by Fz or Dvl (Figure 1E and not shown). Therefore, Fz activation of RhoA requires Dvl but not β-catenin, and is not a secondary effect due to β-catenin signaling.

These results provide direct evidence that RhoA is downstream of Wnt/Fz/Dsh signaling. We note that although the DEP domain of Dvl is required and sufficient for JNK (Jun N-terminal kinase) activation (Boutros et al., 1998; Li et al., 1999), it is not able to activate RhoA (Figure 1E), suggesting that JNK activation by Dsh is independent of RhoA.

A Formin-Homology (FH) Protein Daam1 that Binds to Dvl

Because Dvl2 PDZ domain is required for Fz/Dvl signaling to Rho, we searched, via a yeast two-hybrid screen, for proteins associated with the PDZ domain. We isolated two partial cDNAs that encode overlapping carboxyl terminal fragments (Figure 2E) of a novel protein, referred to as Daam1. The widely expressed human Daam1 protein contains 1078 amino acids (Supplemental Figure S1 at http://www.cell.com/cgi/content/full/107/7/843/DC1), and belongs to the family of Formin homology (FH) proteins that have been implicated in cell polarity from yeast to human (Wasserman, 1998). Formin is the product of the *limb deformity* locus and required for limb morphogenesis in mice (Woychik et al., 1990). Daam1 shares 22% to 30% identity with, and thus is distantly related to, several known mammalian FH proteins. Like other FH proteins, Daam1 contains a central proline-rich FH1 domain and a more carboxyl FH2 domain, and represents a novel subfamily that includes a closely related Daam2, Xenopus and zebrafish Daam, and a Drosophila ortholog dDaam. The Daam subfamily exhibits extensive similarity both within and outside the FH1 and FH2 domains, including the amino and carboxyl terminal regions (Supplemental Figure S1). Since several FH proteins bind to Rho, Rac, or Cdc42 (Wasserman, 1998), Daam1 may also bind Rho GTPases.

Daam1 Mediates Wnt-Induced Dvl-Rho Complex Formation

We first examined whether Daam1 interacts with Dvl using epitope-tagged Dvl2, Daam1, or their mutant forms (Figures 1D and 2E). Daam1 binds to both PDZ and DEP domains, which are required for PCP signaling, but not to the DIX domain (Figure 2A). Consequently, Daam1 binds to Dvl2, ΔDIX-Dvl, ΔPDZ-Dvl, or ΔDEP-Dvl (Figure 2C and not shown). The yeast two-hybrid screen suggests that Daam1 carboxyl terminal region is responsible for Dvl binding. Indeed, C-Daam1, which contains the tail region plus the FH1 and FH2 domains (Figure 2E), interacts with Dvl2 (Figures 2A and 2D), whereas N-Daam1, which is the amino terminal quarter (Figure 2E), does not (Figure 2D). Daam1 binding to Dvl is specific, because another FH protein, mDia2, or its mutant ΔGBD-mDia2, which lacks the amino terminus and thus is analogous to C-Daam1 (Tominaga et al., 2000), does not interact with Dvl2 (Figure 2B), reflecting distinct carboxyl terminal regions of Daam1 and mDia2.

We next examined whether Daam1, like some FH proteins, binds to Rho GTPases. Daam1 or N-Daam1, but not C-Daam1, binds to both GDP and GTP-bound RhoA in vitro, with stronger binding toward GTP-bound RhoA (Figure 2F). Daam1 exhibits little, if any, binding to Rac or Cdc42, whereas PAK (p21 activated kinase) binds to either GTP-loaded Rac or Cdc42, but not Rho (Figure 2F). These results suggest that Daam1 contains a Rho binding domain at its amino terminal region.

Since Daam1 binds to Dvl and RhoA, we tested whether Daam1 forms a ternary complex with Dvl and RhoA. We immunoprecipitated the endogenous RhoA from 293T cells treated with or without Wnt-1 CM. Strikingly, we found that the endogenous Dvl protein is associated with RhoA only upon Wnt stimulation (Figure 2G). The Wnt induced Dvl-RhoA complex requires the endogenous Daam1 protein, because this complex is abolished (Figure 2G) by the expression of either N-Daam1, which binds RhoA but not Dvl, or T-Daam1, which is a fragment from the two-hybrid screen (Figure 2E) and thus binds Dvl but not RhoA. These results suggest that Wnt signaling induces a Dvl-Rho complex formation that is assembled via Daam1.

Daam1 Is Required for Wnt/Fz/Dvl Activation of Rho

We next examined whether Daam1 function is required for Rho activation by Wnt/Fz/Dvl signaling. Since either N-Daam1 or T-Daam1 disrupts the Dvl-Rho complex formation, they may represent dominant negative Daam1 mutants. Indeed, either N-Daam1 or T-Daam1, which do not induce RhoA activation, blocks RhoA activation by Wnt-1 CM, Fz, or Dvl, but not by Ephexin (Figures 3A and 3B and not shown), suggesting that Daam1 function

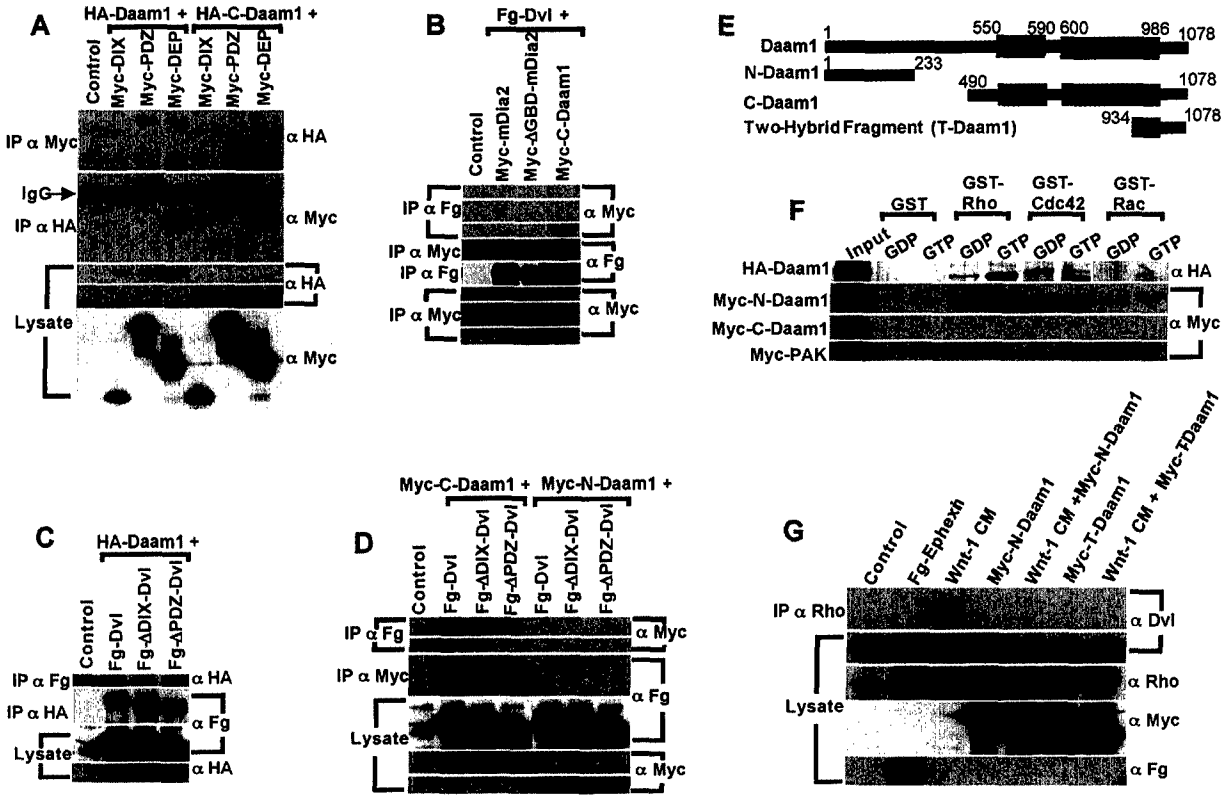

Figure 2. Wnt Induction of a Dvl-Rho Complex Assembled via Daam1

(A–D) Coimmunoprecipitation. Plasmids for tagged Dvl and Daam1 or their mutants were cotransfected, and cell lysates were immunoprecipitated (IP) with indicated Abs. Precipitates were then immunoblotted with indicated Abs (shown on the right side). (A) Daam1 and C-Daam1 bind to the Dvl2 PDZ and DEP domains, but not to the DIX domain. (B) C-Daam1, but neither mDia2 nor ΔGBD-mDia2, binds to Dvl2. (C and D) Daam1 and C-Daam1, but not N-Daam1, bind to Dvl2, ΔDIX-Dvl, or ΔPDZ-Dvl.

(E) Daam1 and mutants. Numbers indicate amino acid positions. T-Daam1 corresponds to one of the two fragments from the yeast two-hybrid screen (the other starts from amino acid position 941).

(F) Daam1 and N-Daam1 interact with GST-Rho-GDP and GST-Rho-GTP in vitro. Lysates from cells transfected with cDNAs for Daam1, its mutants, and PAK were precipitated with GST, GST-Rho, -Rac, or -Cdc42 preloaded with GDP or GTP, and bound Daam1, mutants, or PAK were detected via immunoblotting. (G) Wnt-1 CM induces the endogenous Dvl-RhoA complex formation, which is abolished by either N-Daam1 or T-Daam1 expression. Ephexin, which activates RhoA, does not induce Dvl-RhoA association. Rho was immunoprecipated, and coprecipitated Dvl was detected via immunoblotting via Dvl Abs. Cells were treated with Wnt-1 CM for 3 hours.

is specifically required downstream of Dvl in Wnt/Fz activation of Rho. We also employed double-strand (ds) RNA mediated interference (RNAi) (Elbashir et al., 2001) to deplete endogenous Daam1 protein. The ds RNAi oligo for Daam1 reduces the protein level of Daam1, but not of RhoA, β-catenin (Figure 3C) or Dvl2 (not shown). Importantly, the RNAi oligo inhibits RhoA activation by Wnt-1, Fz, or Dvl, but not by Ephexin (Figure 3C). A control ds RNAi oligo has no effect on Daam1 or RhoA protein level or RhoA activation (Figure 3C). Thus, Daam1 is essential for Wnt/Fz/Dvl activation of RhoA.

An Activated Daam1 Induces Rho Activation in a Rho-GEF-Dependent Manner

Previous studies suggest that deletion of the amino terminal domain results in constitutive activation of FH proteins by releasing an intramolecular inhibition (Watanabe et al., 1999; Tominaga et al., 2000; Westendorf, 2001). C-Daam1 thus may represent an activated form of Daam1. Indeed, while the full length Daam1 is inactive, C-Daam1 overexpression causes activation of RhoA, but not of Rac or Cdc42 (Figure 3D). C-Daam1 activation of RhoA is not affected by ΔPDZ-Dvl (Figure 3A), which inhibits RhoA activation by Fz or Dvl (Figure 1F), further demonstrating that Daam1 functions downstream of Dvl in Rho activation.

Rho, Rac, and Cdc42 elicit distinct morphological changes in cultured mammalian cells (Hall, 1998). Specifically, Rho activation induces stress fiber formation. We found that N-Daam1 disrupts, whereas C-Daam1 enhances, the formation of actin stress fibers (Figure 4A). Thus, the cell biological assay is consistent with the biochemical evidence that Daam1 regulates RhoA activity.

We next examined the potential mechanism of RhoA activation by C-Daam1. GEFs such as Ephexin are a major class of Rho activators (Hall, 1998; Kaibuchi et al., 1999). It is known that dominant negative GTPase mutants, such as RhoA-N19, Rac-N17, and Cdc42-N17, bind tightly to and thereby titrate specific GEFs, and thus can serve as indicators for GEF-dependent processes (Hart et al., 1994). We found that RhoA-N19, but neither Rac-N17 nor Cdc42-N17, blocks RhoA activation by

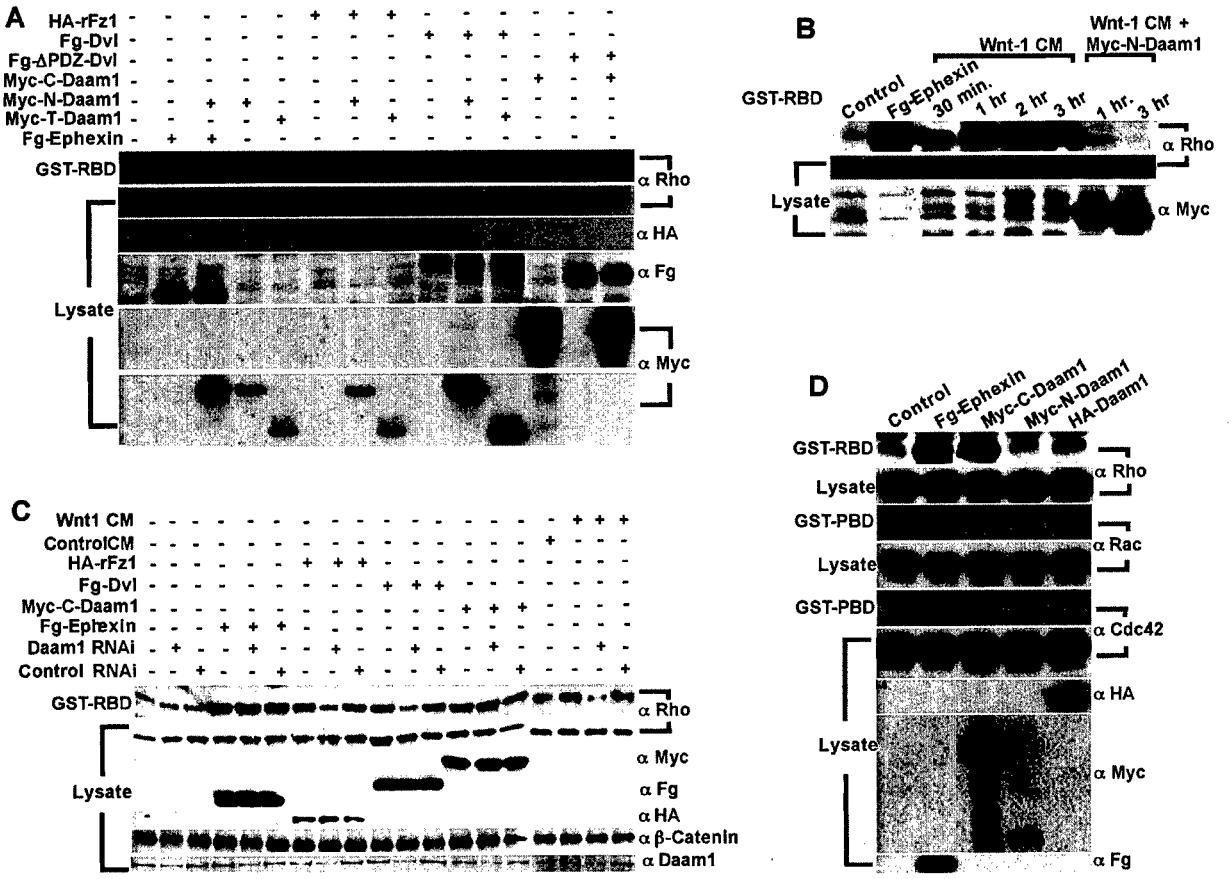

Figure 3. Daam1 Is Required for Rho Activation by Wnt/Fz/Dvl but Not by Ephexin

(A–C) Daam1 functions specifically downstream of Dvl in Rho activation. (A) Either N-Daam1 or T-Daam1 blocks RhoA activation by Fz or Dvl2 but not by Ephexin. ΔPDZ-Dvl2 does not inhibit RhoA activation by C-Daam1. (B) N-Daam1 blocks RhoA activation by Wnt-1 CM. (C) The Daam1 RNAi oligo, but not a control RNAi oligo, inhibits RhoA activation by Wnt-1 CM, Fz, or Dvl, but not by Ephexin or C-Daam1. Daam1 RNAi, but not the control RNAi, reduces the endogenous Daam1 protein to 36 ± 8% of the control level (24 samples of 4 independent experiments), but has no effect on the level of the endogenous RhoA, β-catenin, or Dvl2 protein (not shown), or of the cotransfected cDNA gene products. Note that the C-Daam1 mRNA does not contain the sequence to which the Daam1 RNAi oligo matches, and C-Daam1 protein synthesis is thus resistant to RNAi.

(D) C-Daam1 activates RhoA, but not Rac or Cdc42. The wild type Daam1 activates none.

Wnt/Fz/Dvl, and, importantly, C-Daam1 (Figure 4B). For comparison, RhoA-N19, Rac-N17, and Cdc42-N17 all inhibit Ephexin activation of RhoA (Figure 4B), and of Rac and Cdc42 (not shown), consistent with Ephexin being a GEF for Rho, Rac, and Cdc42 (Shamah et al., 2001). Thus, C-Daam1 activation of RhoA depends on a GEF activity that appears Rho-specific.

As C-Daam1 does not harbor an identifiable GEF motif, we asked whether C-Daam1 might recruit a Rho-GEF. Intriguingly, C-Daam1 in cell extracts is precipitated with GST-RhoA-N19, but barely with GST-Rac-N17 or -Cdc42-N17 (Figure 4C). Because C-Daam1 does not associate detectably with Rho-GTP or Rho-GDP (Figure 2F), these results are indicative of a C-Daam1-associated Rho-GEF, which favors nucleotide-free Rho mimicked by Rho-N19. For comparison, Ephexin is precipitated effectively by GST-Rho-N19, -Rac-N17, -Cdc42-N17, and by GST-Rho, -Rac, and -Cdc42 to a lesser extent (Figure 4C), consistent with Ephexin being a GEF for Rho, Rac, and Cdc42. Thus, C-Daam1 appears to recruit a Rho-GEF.

We also found that an activated form of mDia2, ΔGBD-mDia2 (Tominaga et al., 2000), induces RhoA activation as well (Figure 4D). Thus, FH proteins may activate Rho in other signaling pathways.

Daam1 Mediates Rho Activation by Wnt-11 during Xenopus Gastrulation

To elucidate Daam1 function in vivo, we examined whether Xenopus Daam1, which is expressed throughout embryogenesis (Figure 5A), mediates Wnt-11 PCP signaling in regulating gastrulation movements. Wnt-11 expression and morphogenetic movements occur predominantly in dorsal tissues. Consistently, we found that RhoA is activated in the *d*orsal, but not the *v*entral, *m*arginal *z*one (DMZ versus VMZ) in gastrula embryos (Figure 5B). Wnt-11/Fz PCP signaling is essential for dorsal RhoA activation, because various dominant negative mutants that inhibit Wnt-11 signaling, such as Dn-Xwnt-11 (Tada and Smith, 2000), extra-xFz7 (Djiane et al., 2000), Xdd1 (Sokol, 1996), and importantly, N-Daam1, each prevent RhoA activation in DMZ (Figure 5B). Conversely, activation of Wnt-11 PCP signaling by the ex-

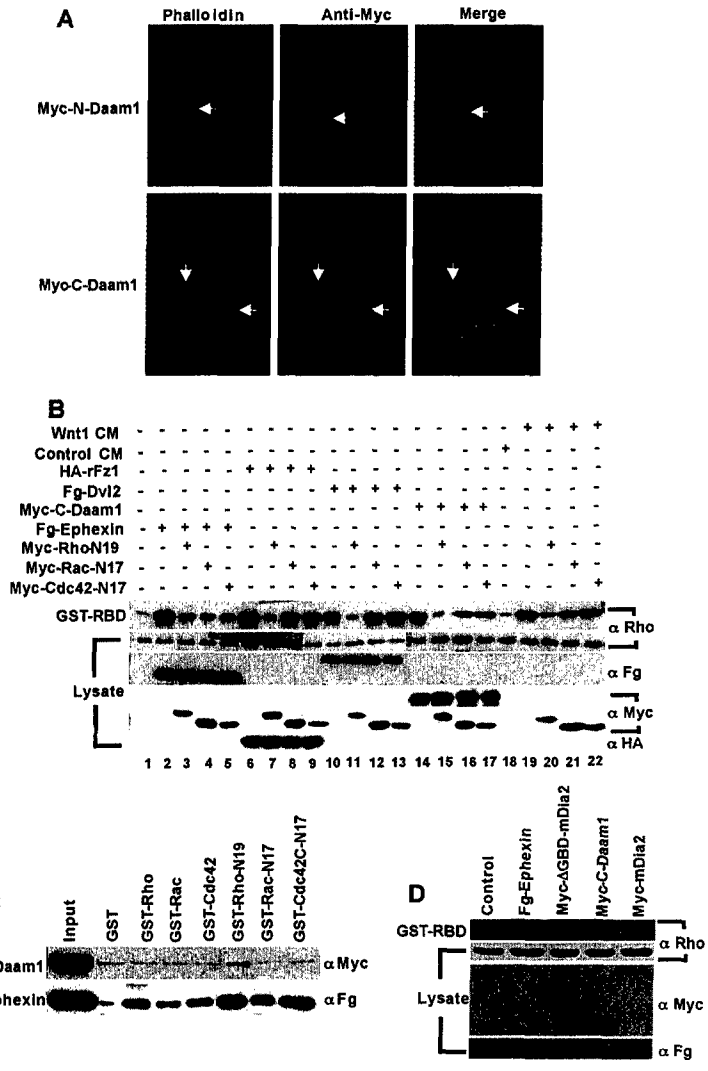

Figure 4. C-Daam1 Affects Stress Fiber Formation and Depends on, and May Associate with, a Specific Rho-GEF for Rho Activation

(A) N-Daam1 collapses, while C-Daam1 induces the formation of actin stress fibers (62 ± 6% and 60 ± 4% of transfected cells, respectively), as visualized with Phalloidin in HeLa cells. Arrows indicate transfected cells.

(B) Rho-N19, but neither Rac-N17 nor Cdc42-N17, blocks RhoA activation by Wnt, Fz, Dvl, or C-Daam1. Rho-N19, Rac-N17, and Cdc42-N17 all inhibit Ephexin activation of RhoA, and of Rac and Cdc42 (not shown).

(C) C-Daam1 in extracts of transfected cells associates with GST-Rho-N19, but not with GST-Rac-N17 or GST-Cdc42-N17, nor with GST-Rho, -Rac, or -Cdc42 (which are likely GDP-bound). Ephexin associates with all the above, but not with GST alone. "Input" represents 1% of C-Daam1 and 10% of Ephexin in extracts incubated with GST-fusion proteins. The low percentage of C-Daam1 precipitated with GST-Rho-N19 may reflect the limiting amount of the putative Rho-GEF associated with C-Daam1.

(D) ΔGDB-mDia2, but not mDia2, activates RhoA.

pression of Xwnt-11, Fz7, or Xdsh, and importantly, C-Daam1 is sufficient to activate RhoA in VMZ (Figure 5C). These results indicate that Wnt-11/Fz PCP signaling, mediated by Xdsh and XDaam1, controls RhoA activity during gastrulation.

Daam1 Regulates Xenopus Gastrulation

To study whether XDaam1 regulates gastrulation, we first examined activin-treated animal pole explants, which represent dorsal tissue and exhibit morphogenetic elongation characteristic of gastrulation (Figure 5D). This elongation is suppressed by N-Daam1, and by Dn-Xwnt-11, extra-xFz7, or Xdd1 as reported (Figure 5D and Supplemental Table S1 at http://www.cell.com/cgi/content/full/107/7/843/DC1). While C-Daam1 alone does not induce elongation (not shown) or affect elongation induced by activin, it fully rescues elongation inhibited by Dn-Xwnt-11, extra-xFz7, or Xdd1 (Figure 5D and Supplemental Table S1). We further employed a Morpholino oligo (MO) that is designed to specifically block endogenous XDaam1 protein synthesis. Inhibition of elongation is observed in explants from embryos injected with the XDaam1 MO, but not a control MO (Figure 5D and Supplemental Table S1). This inhibition is reversed by coinjection with either human Daam1 RNA or C-Daam1 DNA, which direct human Daam1 or C-Daam1 protein synthesis that is resistant to the MO interference (Figure 5D and Supplemental Table S1). Thus, both biochemical and embryological assays suggest that Daam1 functions downstream of Dvl in the Wnt-11/Fz PCP pathway.

Although N-Daam1 blocks elongation movements, N-Daam1 (or C-Daam1) does not inhibit activin induction of mesodermal genes, such as *Brachyury* (*Xbra*), *Goosecoid* (*Gsc*), *Chordin*, and *Xwnt-8* (Figure 5E), indicating that Daam1, like Wnt-11, XFz7, or Xdsh, regulates gastrulation movements without interfering with mesoderm formation (Sokol, 2000). In addition, Daam1, N-Daam1, or C-Daam1 neither induces nor affects Xwnt-8 induction of *Siamois* and *Xnr3* (Figure 5F and not shown), which are activated by Wnt/β-catenin signaling. Therefore, Daam1 function is specifically required for Wnt/Fz PCP but not Wnt/β-catenin signaling.

We further investigated Daam1 function during gastrulation in vivo. Embryos injected dorsally with N-Daam1 RNA develop normally prior to gastrulation (Figure 6A). But from the onset of gastrulation, these embryos exhibit

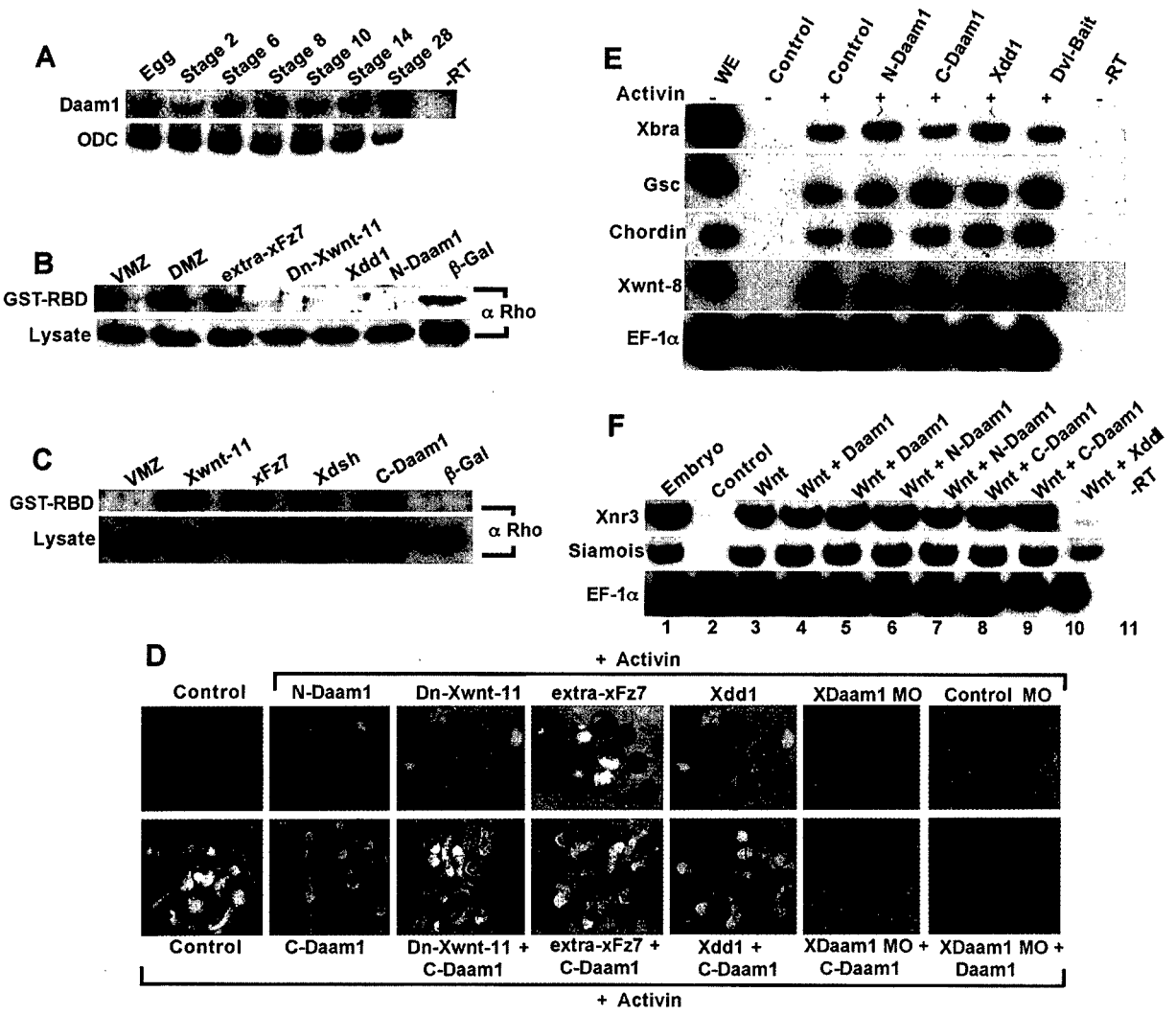

Figure 5. Daam1 Is Required for Dorsal RhoA Activation and Morphogenetic Movements of Animal Pole Explants

(A) Xenopus Daam1 is expressed maternally and throughout embryogenesis as monitored by RT-PCR. ODC is used as an internal control. −RT: without reverse transcriptase. In situ hybridization suggests Daam1 expression throughout embryogenesis (not shown).

(B) Endogenous RhoA activation is detected in DMZ but not in VMZ at stage 10.5, and is inhibited in DMZ by RNAs for Dn-Xwnt-11 (1 ng), extra-xFz7 (1 ng), Xdd1 (1 ng), or N-Daam1 (2 ng), but not by β-galactosidase (β-gal) RNA (2 ng).

(C) RhoA is activated in VMZ by RNAs for Xwnt-11 (200 pg), xFz7 (200 pg), or Xdsh (1 ng), or C-Daam1 DNA (200 pg), but not by β-gal RNA.

(D) In animal pole explants treated with activin, N-Daam1 RNA (2 ng) inhibits elongation, whereas C-Daam1 DNA (200 pg) rescues elongation inhibited by RNAs for Dn-Xwnt-11, extra-xFz7, and Xdd1, or by the XDaam1 MO (40 ng). Human Daam1 RNA (2 ng) also rescues the inhibition by XDaam1 MO.

(E) Neither N-Daam1 nor C-Daam1 affects mesodermal gene induction by activin in animal pole explants, as assayed by RT-PCR at stage 10.5. EF-1α was used as an internal control. WE: whole embryo.

(F) RNA for Daam1 or N-Daam1 (1 or 2 ng), or C-Daam1 DNA (100 or 200 pg), does not affect Xwnt-8 (20 pg) induction of *Xnr3* and *Siamois* in animal pole explants at stage 10.5. Xdd1 RNA (1 ng) inhibits *Siamois* and *Xnr3* induction by Xwnt-8, reflecting dual roles of Xdsh in β-catenin and PCP signaling.

severe morphogenetic defects manifested by a large and open blastopore, exposed endoderm cells, and a shortened body axis and microcephaly (Figure 6A and Supplemental Table S2 at http://www.cell.com/cgi/content/full/107/7/843/DC1). Characteristic of Fz PCP signaling (Tada and Smith, 2000; Wallingford et al., 2000; Djiane et al., 2000), embryos injected dorsally with C-Daam1 also exhibit severe gastrulation abnormalities (Supplemental Table S2). Importantly, embryos injected with the XDaam1 MO, but not the control MO, show similar gastrulation defects, which are rescued by coinjection with human Daam1 RNA (Figure 6A and Supplemental Table S2). Embryos injected ventrally with N-Daam1 RNA or the XDaam1 MO develop rather normally (not shown). Using mesoendodermal and neural markers, we found that embryos injected dorsally with N-Daam1 RNA, while expressing normal levels of specific marker genes, exhibit defects in positioning and morphology of mesoendodermal and neural tissues (Figure 6B). These phenotypes, reflecting normal cell fate specification but abnormal gastrulation, are similar to those resulted from Dn-Xwnt-11, extra-xFz7, or Xdd1,

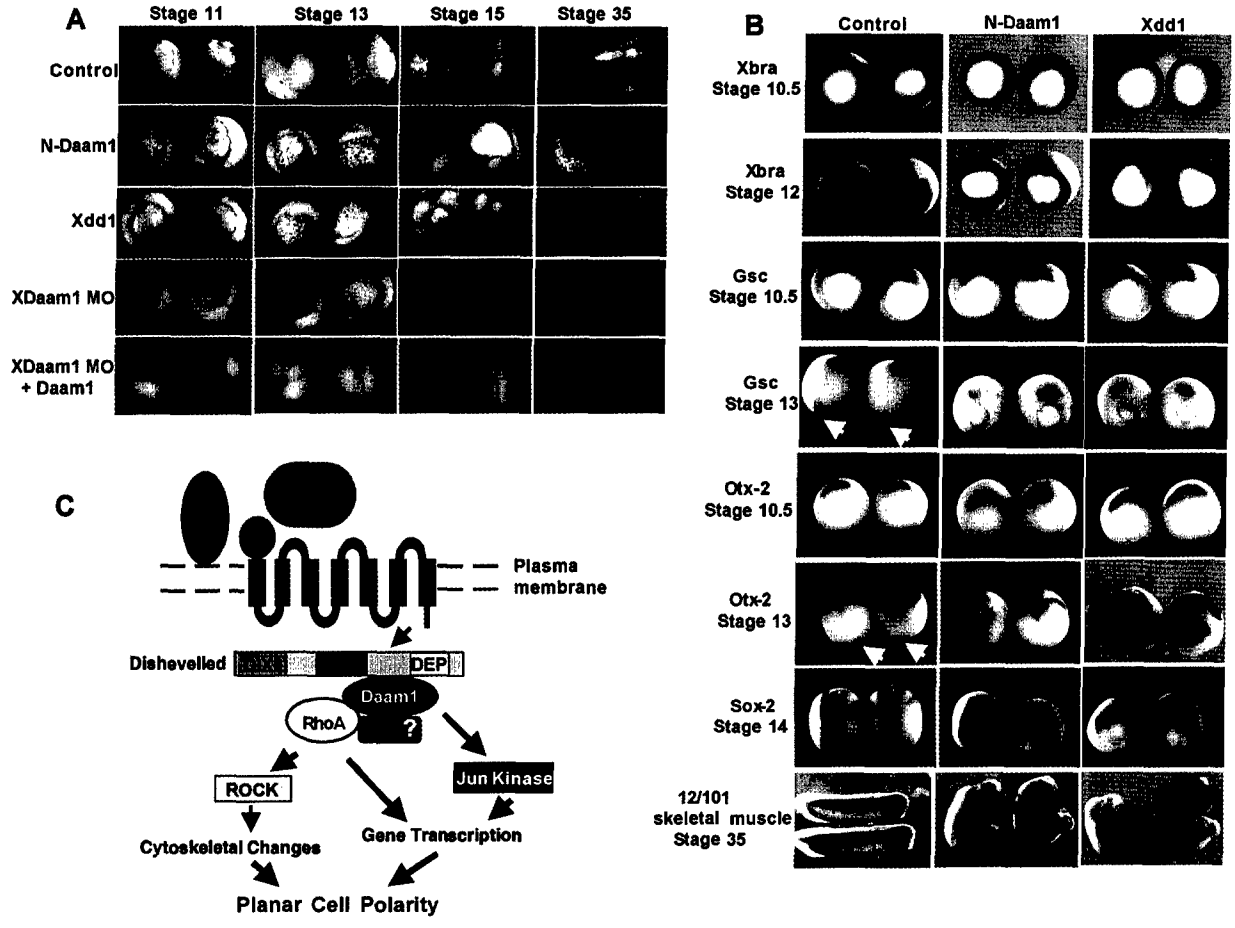

Figure 6. An Essential Role of Daam1 in Gastrulation and in the Wnt/Rho Signaling Pathway

(A) N-Daam1 or XDaam1 MO inhibits gastrulation. RNA (2 ng) for N-Daam1 or Xdd1 (for comparison) or Xdaam1 MO (40 ng) was injected dorsally at 4-cell stage (st). XDaam1 MO effect was rescued by coinjection with human Daam1 (2 ng), but not β-gal (2 ng) RNA.

(B) Embryos injected dorsally with N-Daam1 or Xdd1 RNA. N-Daam1 injected embryos show normal expression of mesodermal Xbra, and dorsal Gsc and Otx2 at st 10.5, but exhibit an Xbra expression surrounding a large-sized blastopore and missing from the involuting notochord at st 12. Gsc expression in control embryos at st 13 is observed in anterior mesoendoderm far from the closed blastopore (arrowheads), but remains in a dorsal region close to the open blastopore in N-Daam1 injected embryo. Otx-2 is expressed anteriorly in both mesodermal and overlying neural tissues in control embyos at st 13. In N-Daam1 injected embryos, two separate Otx-2 expression domains are obvious. A bisection shows that the dorsal margin expression near the blastopore reflects the anterior mesoderm that fails to involute, and the other is in neuroectoderm. Sox-2 is expressed in the neural plate at st 14. In N-Daam1 injected embryos, Sox-2 expression is seen in a broad dorsal region that surrounds the open blastopore and lacks neural plate morphology. Finally, mAb 12/101, which is muscle-specific, detects normal amount of muscle formation along a severe shortened/bent body axis in N-Daam1 injected embryos. N-Daam1 phenocopies Xdd1 in all cases.

(C) A model for the Wnt/Rho signaling pathway (see Discussion).

demonstrating an essential role of Daam1 in Wnt-11 PCP signaling during gastrulation.

Discussion

We demonstrated that Wnt/Fz signaling via Dvl activates RhoA, a key regulator of cell polarity. We identified a novel FH protein, Daam1, which mediates Wnt/Fz activation of RhoA via the assembly of a Wnt-induced Dvl-RhoA complex. We further showed that Daam1 is essential for Wnt/Fz activation of RhoA and for Xenopus gastrulation. These findings elucidate a molecular pathway linking Wnt signaling to Rho in governing cytoskeletal architecture and polarity.

Wnt/Fz Signaling Activates RhoA

Genetic studies have implicated DFz1, Dsh, RhoA, Rac, Drok (ROCK), and several other cytoplasmic and transmembrane proteins in PCP establishment in Drosophila (Shulman et al., 1998; Mlodzik, 1999; Winter et al., 2001). While DFz1, Dsh, and a seven-pass transmembrane cadherin (Flamingo) are interdependent on one another for polarized localization (Strutt, 2001; Axelrod, 2001; Shimada et al., 2001), whether/how PCP gene products function in a common pathway or multiple parallel pathways is unknown. Our results that Fz/Dvl signaling activates RhoA thus provide a direct biochemical link from Fz signaling to Rho. Fz activation of RhoA requires Dsh/Dvl but not β-catenin (Figure 1E), corroborating with the genetic evidence that Fz/Dsh PCP and Wnt/β-catenin signaling are distinct pathways (Axelrod et al., 1998; Boutros et al., 1998). Although genetic analyses have not implicated Wnt signaling in Fz PCP or Rho GTPase regulation, we demonstrated that Wnt-1 and Wnt-11 activate RhoA in human and Xenopus, in a manner independent of new protein synthesis (Figures 1B, 1C, 5B,

and 5C). Thus, Rho activation is a direct Wnt response and likely represents a key pathway for Wnt regulation of cytoskeleton. Wnt/Fz/Dvl activation of RhoA in 293T cells recapitulates many aspects of PCP signaling in embryos, establishing a simple cell model for Wnt/Fz PCP signaling that has been previously refractory to biochemical analyses.

Wnt-11 is implicated in PCP signaling, and indeed induces and is required for RhoA activation during gastrulation. Wnt-1 activation of RhoA is unexpected, however, as Wnt-1 has thus far been associated only with β-catenin signaling. Wnt-1 and certain Fz receptors activate both Rho and β-catenin signaling (Figures 1A–1C), raising the possibility that Wnt-1 (and perhaps other Wnt) functions are mediated by Rho and β-catenin. Several observations are consistent with this notion. First, Wingless (Wnt-1) is required for cell shape changes during Drosophila dorsal closure in a manner that is distinct from β-catenin (McEwen et al., 2000). Second, mammary cells transformed by Wnt-1 show morphological changes, which often reflect activities of Rho GTPases. Third, a stabilized β-catenin does not fully mimic Wnt-1 effects in fibroblasts (Young et al., 1998). Finally, Wnt-1, but not β-catenin, induces the expression of *Wrch-1*, which encodes a Cdc42-related molecule (Tao et al., 2001). Whether Wnt-1 induction of *Wrch-1* is mediated via RhoA remains to be examined.

Daam1: An FH Protein Required for Wnt/Fz Activation of RhoA

We identified a novel Dvl-binding protein, Daam1, a member of FH proteins that have been implicated in cell polarity in eukaryotes (Wasserman, 1998). Daam1 binds to Dvl and RhoA, but not to Rac or Cdc42 (Figures 2A–2F and 4C). Importantly, Wnt signaling induces the formation of a Dvl-RhoA complex that is assembled by Daam1 (Figure 2G). Interference of Daam1 function via Daam1 mutants that disrupt Wnt-induced Dvl-RhoA complex formation, or depletion of Daam1 protein via RNAi, inhibits RhoA activation by Wnt/Fz/Dvl, but not by Ephexin (Figures 3A–3C). Thus Daam1 is specifically required downstream of Dvl in Wnt/Fz activation of RhoA.

Overexpression of C-Daam1, which lacks the amino terminal region and appears constitutively active, induces RhoA, but not Rac or Cdc42, activation (Figure 3D). C-Daam1 activation of RhoA depends on a Rho-GEF, as it is blocked by Rho-N19 (Figure 4B). C-Daam1 is found in a complex with Rho-N19, but not with Rho-GDP or Rho-GTP, nor with any forms of Rac or Cdc42 (Figures 2F and 4C). Thus, C-Daam1 may recruit a Rho-GEF. Another activated FH protein also causes RhoA activation (Figure 4D), suggesting that at least some FH proteins have a capability to promote Rho activation, likely mediated by the conserved FH1 and FH2 domains. FH1 is proline-rich and can interact with Src-homology 3 (SH3) and WW motifs (which mediate protein interactions), the actin-binding protein profilin (Wasserman, 1998), the Src kinase (via SH3) (Tominaga et al., 2000), and Rac (Westendorf, 2001), and possibly other proteins. No binding proteins for FH2 are yet known. It will be critical to identify proteins, including a putative Rho-GEF associated with C-Daam1.

The activated C-Daam1 reveals an autoinhibition mechanism similar to that found in other FH proteins, which are maintained in an inactive state by an intramolecular binding between the amino terminal region and the carboxyl autoregulatory domain (Watanabe et al., 1999; Nakano et al., 1999; Alberts, 2001; Westendorf, 2001). Dvl, which specifically binds to the carboxyl terminus of Daam1 but not of another FH protein (Figure 2B), likely releases such an intramolecular inhibition, thereby allowing Daam1 to function in RhoA activation. Consistent with this notion, expression of the Daam1 carboxyl terminus (T-Daam1) prevents Dvl activation of RhoA (Figure 3A).

The Daam1 amino terminus binds to Rho-GDP or Rho-GTP (Figure 2F), suggesting a role for Daam1 as a scaffolding protein to recruit Rho-GDP (via the amino terminus) and a Rho-GEF (via the C-Daam1 portion), thereby enhancing Rho-GTP formation. The Daam1 amino terminus binds Rho-GTP with apparently higher affinity, raising an intriguing possibility of positive feedback control, a theme common in cell polarization. Polarity establishment relies on signal amplifications that interpret a small difference in a polarity signal field into a polarized cellular response (Gulli and Peter, 2001). DFz1 exhibits a polarized localization that depends on Dsh function (Strutt, 2001; Axelrod, 2001), suggesting a positive feedback loop. Rho-GTP binding to the Daam1 amino terminus may stabilize Daam1 in its activated state, or recruit/activate additional Daam1, thereby promoting an amplification of Rho activation. Such a feedback loop would resemble one in pheromone-induced polarity in yeast. The mating pheromone, via its serpentine receptor and the trimeric G protein, recruits and activates a GEF specific for Cdc42. Activated Cdc42, in turn, is required for the GEF localization, thereby leading to further and polarized Cdc42 activation (Gulli and Peter, 2001). We note that we cannot rule out the possibility that Daam1 may function primarily in such a feedback control. In this scenario, Wnt/Fz signaling initiates Rho activation without Daam1, and the activated Rho together with Dvl recruits/activates Daam1 to amplify Rho activation. In any event, Daam1 function is essential for Rho activation triggered by Wnt/Fz signaling.

Daam1 is distantly related to several distinct mammalian FH proteins, such as FRL (Yayoshi-Yamamoto et al., 2000) (30% identity), FHOS (Westendorf et al., 1999) (27%), mDia1 (Watanabe et al., 1997) (28%), and mDia2 (Tominaga et al., 2000) (22%), whose functions in GTPase signaling remain to be fully understood. FRL and FHOS bind specifically to Rac in a nucleotide-independent manner, and an activated FHOS is antagonized by Rac and Rac mutants, leading to the suggestion that FRL and FHOS are scaffolding proteins linking Rac to other proteins (Yayoshi-Yamamoto et al., 2000; Westendorf, 2001). The mDia subfamily of FH proteins bind to Rho-GTP (and Rho-GDP in some cases), and are proposed to be Rho targets (Nakano et al., 1999; Watanabe et al., 1999; Tominaga et al., 2000). However, as actin fiber induction by the activated mDia can be blocked by inhibition of Rho in some instances (Nakano et al., 1999), and the activated mDia can cause RhoA activation (Figure 4D), the relationship between mDia and Rho, and between FH proteins and Rho GTPases in general, may be complex and needs further investigation.

Daam1 Is Required for Wnt/Fz PCP Signaling during Gastrulation

Vertebrate gastrulation involves polarization and intercalation of dorsal mesodermal cells along the mediolateral axis (convergence), resulting in the elongation of the anterioposterior axis (extension). This morphogenetic process is governed by Wnt-11 PCP signaling (Tada and Smith, 2000; Heisenberg et al., 2000; Wallingford et al., 2000). We found that in Xenopus gastrula, endogenous Rho activation is detected mainly in dorsal tissue, and is abolished when Wnt-11/Fz/Xdsh signaling or Daam1 function is inhibited. Conversely, ectopic Wnt-11/Fz/Xdsh signaling or C-Daam1 activates RhoA on the ventral side (Figures 5B and 5C). Thus, Wnt-11/Fz signaling, via Xdsh and Daam1, is necessary and sufficient for RhoA activation during gastrulation, consistent with the previous finding that interference of Rho function inhibits gastrulation (Wunnenberg-Stapleton et al., 1999). In an explant assay, inhibition or depletion of Daam1 perturbs morphogenetic movements, whereas C-Daam1 restores the movements even when Wnt-11/Fz or Xdsh is inhibited (Figure 5D). Daam1 thus functions downstream of Wnt-11/Fz/Xdsh in governing gastrulation. Finally, inhibition or depletion of Daam1 in the embryo blocks gastrulation and phenocopies the morphogenetic defects caused by inhibition of Wnt-11, Fz, or Xdsh signaling (Figures 6A–6B).

A Wnt/Rho Signaling Pathway

We suggest a molecular pathway for Wnt/Fz activation of Rho (Figure 6C), which we refer to as the Wnt/Rho pathway to distinguish it molecularly from Wnt/β-catenin and Wnt/Ca^{2+} pathways (Wodarz and Nusse, 1998; Miller et al., 1999). A Wnt signal activates a Fz receptor, which translocates Dsh to the plasma membrane and promotes Dsh-Daam1-RhoA complex formation and RhoA activation, likely via the recruitment of a RhoGEF by the Daam1 scaffolding protein. Activated RhoA generates polarized cytoskeleton remodeling via the ROCK kinase (Winter et al., 2001), and perhaps also induces changes in gene expression (Fanto et al., 2000). The zebrafish *knypek* gene product, a glypican, facilitates Wnt signal reception (Topczewski et al., 2001), whereas LRP5/6, which is the Fz coreceptor for Wnt/β-catenin signaling, participates in neither PCP signaling (Wehrli et al., 2000; Semenov et al., 2001) nor RhoA activation (not shown). Whether and how other PCP gene products function in the Wnt/Rho pathway or in parallel pathways remain to be elucidated.

Experimental Procedures

Antibodies

Monoclonal antibodies (mAbs) against HA (F-7), RhoA (26C4), Dvl2 (10B5), and Myc (9E10), and polyclonal Abs (pAbs) against RhoA (CAT119) and Myc (N-262), were from Santa Cruz Biotechnology. mAbs against Rac and Cdc42 were from Transduction Laboratories, and against Flag (M2) was from Sigma. Alexa Fluor 488 goat-anti-mouse Ab and Texas Red X-Phalloidin were from Molecular Probes.

Anti-Daam1 antibodies were generated in chickens against a GST fusion protein containing amino acids 967–1078 of the human Daam1. Egg IgY was purified using the Eggcellent Purification Kit (Pierce), and further affinity purified using the GST-Daam1. The final IgY specifically recognizes Daam1 (119 kD) (not shown).

Plasmids and Oligonucleotides

The mouse Dvl2 and mutants were generated by restriction digestions or site-directed mutagenesis, and subcloned in pCS2+ and/or pCS2+MT (for the Myc tag at the amino terminus). The Flag tag was introduced by PCR at the amino terminus. The human Daam1 cDNA was used with the exception of T-Daam1 (rat). N-Daam1, C-Daam1, and T-Daam1 were amplified by PCR, and like Daam1, were cloned into CS2+, CS2+MT, and/or pcDNA-HA (for the HA tag at the amino terminus). Details are available upon request.

The ds RNAi oligo for human Daam1 (and Daam2) were synthesized by Integrated DNA Technologies: 5′-GCAGGCCAUGCUGCA CUACTT-3′ (sense) and 5′-GUAGUGCAGCAUGGCCUGCTT-3′. A ds RNAi oligo for zebrafish squint gene of identical length was used as the negative control.

The XDaam1 MO complementary to the translational initiation site, 5′-GCCGCAGGTCTGTCAGTTGCTTCTA-3′, was synthesized by Gene Tools Inc. A MO with a random sequence was used as the negative control.

Yeast Two-Hybrid Screen

A rat brain cDNA library (Clontech) was screened using mDvl2 PDZ domain (Figure 1D) as the bait. 3.8 million independent clones were screened, 54 positives were obtained, of which two were Daam1 fragments (Figure 3E).

Transfections

All were done with HEK 293T cells except for Figure 4A (HeLa). Cells in a 6-well plate were transfected via the calcium phosphate method with 2 μg of each indicated plasmid with the exception of Rho-N19, Rac-N17, and Cdc42-N17 (3 μg each), or via Lipofectamine (Life Technologies) with 3 μg annealed RNAi oligo plus 2 μg plasmid. Transfected DNA amounts were equalized via vectors without inserts.

Rho, Rac, and Cdc42 Activation and Binding Assays

Cells were lysed 36 hours (or 72 hours for RNAi) posttransfection in the lysis buffer for Rho (Ren et al., 1999) or for Rac/Cdc42 (Benard et al., 1999). Xenopus DMZ or VMZ was dissected at stage 10.5 and lysed in the Rho lysis buffer. GST-RBD and GST-PBD binding assays were performed as described (Ren et al., 1999; Benard et al., 1999), and samples were resolved by 12% SDS-PAGE and immunoblotted with the anti-Rho, -Rac, or -Cdc42 mAb for cell lysates and anti-Rho pAbs for Xenopus explants. Cycloheximide was added at a final concentration of 10 μg/ml, starting one hour prior to Wnt-1 CM treatment.

Extracts of transfected cells were lysed and precipitated by GDP or GTP-loaded GST-Rho, -Rac or -Cdc42 (Figure 2F), or GST-Rho (or N19), -Rac (or N17) -Cdc42 (or N17) (Figure 4C) as described (Lu and Settleman, 1999). Samples were resolved by 12% SDS-PAGE and immunobloted with anti-Myc, -HA, -Flag mAbs.

Immunoprecipitation, Immunoblotting, and Immunocytochemistry

Cells were lysed 36 hours posttransfection in 0.5% NP40 lysis buffer for Dvl/Daam1 interactions or the Rho lysis buffer for Dvl/Rho complex detection. Lysates were precipitated with anti-Myc (pAbs), or anti-Flag, -HA, -Rho mAb, resolved by 12% SDS-PAGE and blotted with anti-Myc, -Flag, -HA, -Rho, or -Dvl2 mAb. Immunocytochemistry (Figure 4A) was performed as described (Tominaga et al., 2000).

Embryo Manipulations, RT-PCR, In Situ Hybridization, and Explant Assays

These were performed as described (Kato et al., 1999). Embryo injections were done with in vitro transcribed RNAs, except for C-Daam1, which was injected as DNA plasmid. Convergent extension assays in explants were performed as described (Sokol, 1996) using 20 ng/ml activin (final).

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